# DETERMI. BACTE
# LAB. MANUAL

H. H. LORD

1. ~~pure~~ cultures
2. ~~gram~~ staining
3. motility
4. bacillaceae
5. lactobacteriaceae
6. enterobacteriaceae
7.

# Determinative Bacteriology
# LABORATORY MANUAL

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# PREFACE

A survey of textbooks in the field of bacteriology has shown a need for text dealing with the procedure for identifying unknown bacteria. Some exts consider a small group of organisms, such as the Enterobacteriaceae or he Streptococceae. The Manual of Methods for Pure Culture Study of Bacteria as excellent material for advanced students and research workers, but it is ardly suitable for classroom use without considerable elaboration by the in-ructor on how to carry out a systematic program for identification of an known bacterium.

This laboratory manual in the elements of determinative bacteriology has ēn compiled to meet the needs for a step-by-step procedure for identifying e common members of the Eubacteriineae encountered as unknown organisms in urses in bacterial identification.

The basic concept of this text is to lead the student through the vari-s, progressive steps of isolation, purification, morphologic study, cul-ral study, and biochemical study of an organism. At all times the student being trained to perfect his skill in routine techniques by repetition on ny different organisms. He thereby gets a better picture of the "individu-ity" of organisms within certain "greater patterns" of shape and behavior.

It is not felt that this manual should cover every known Family of the cteria, hence, only the more common or representative groups are studied in tail.

The manual was designed for a course having three two-hour laboratory ssions per week. The instructor may delete or add experiments to those esented herein to suit his own needs. To accomplish the entire set of periments as presented, it is presumed that the student will not have to e his laboratory class-time in washing glassware or preparing media.

During the course of the laboratory work, reference should be made to the nual of Methods for Pure Culture Study of Bacteria and the 6th Edition of rgey's Manual of Determinative Bacteriology.

Of necessity many fundamental procedures learned in beginning courses ve been repeated to aid the student in refreshing his memory and to give mpleteness to the text. Many procedures are adapted from other well recog-zed texts and manuals in the field of bacteriology, and the author is deep-grateful to those authors and publishers who have given permission to use pyrighted material.

The author is grateful to several whose kind assistance was invaluable ring the publication of this manual. He especially wishes to thank Dr. P. L. iney for many helpful suggestions and a most critical reading of the finished nuscript.

A text of this type invariably seems to have omissions and arrangement ich are awkward for some users. Suggestions from users will be gratefully preciated as an aid to improving the usefulness of the manual.

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Manhattan, Kansas
June, 1951

# TABLE OF CONTENTS

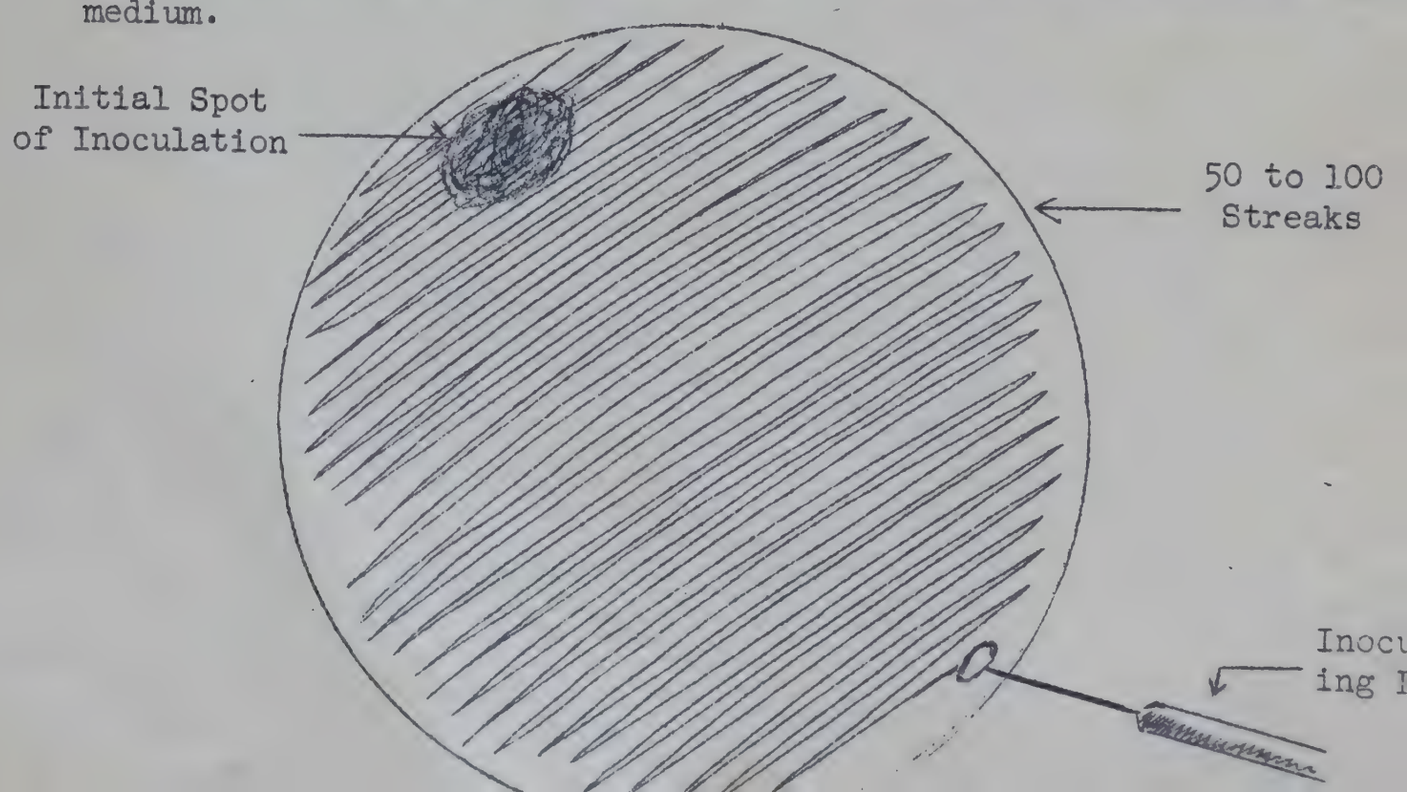
Exercise 1

# ISOLATION TECHNIQUES FOR SECURING PURE CULTURES FROM NATURAL MATERIALS

The first step in making a determinative study of a bacterium from a natural material is to isolate the organism in pure culture upon a suitabl medium. Many methods of isolation are available. The two following metho are satisfactory and have found wide favor.

## First Day: Procedure A: The Streaked Plate Method

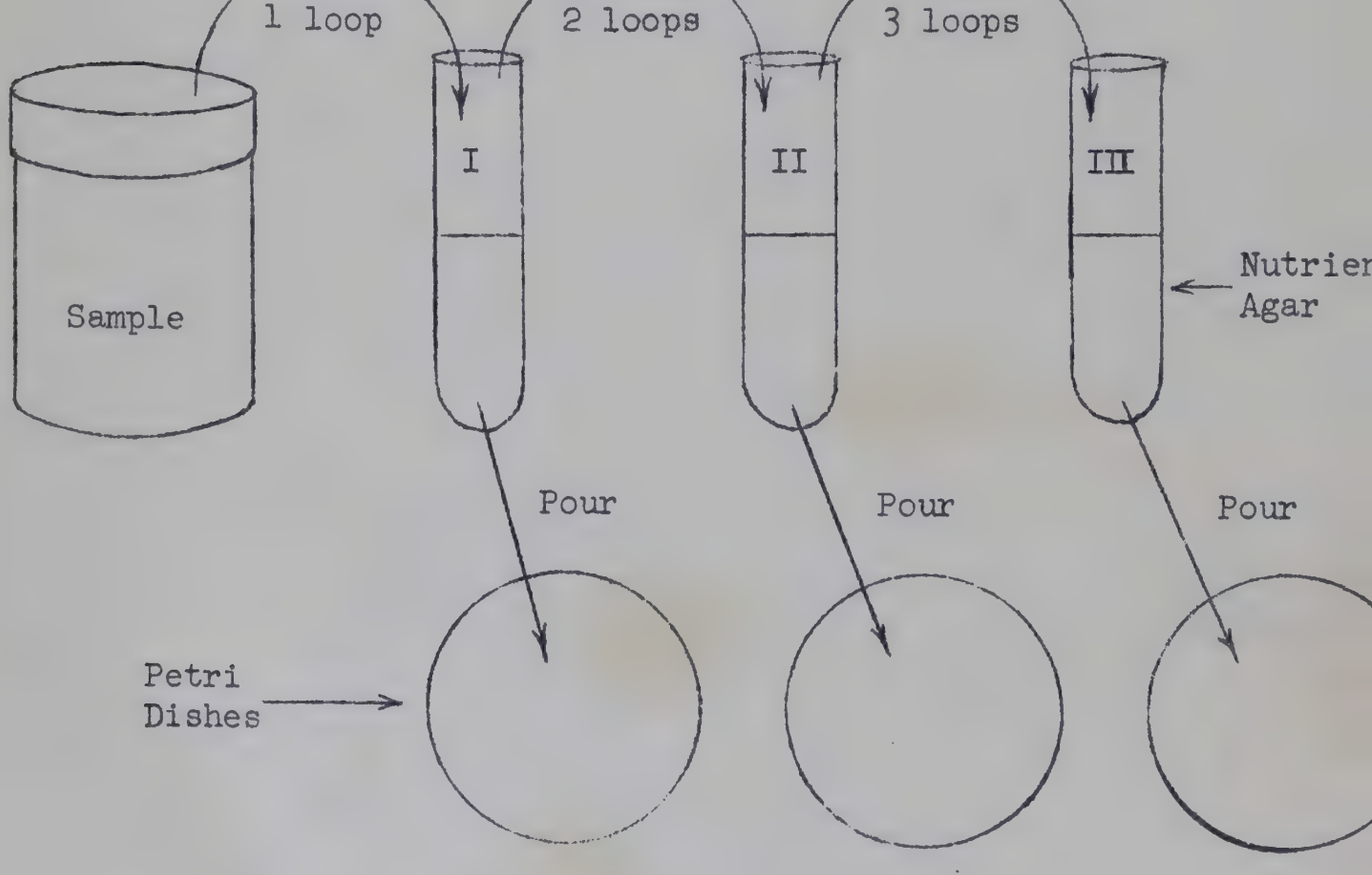
1. Melt a tube of sterile Nutrient Agar medium at 100° C.
2. Cool the melted Nutrient Agar by placing in a water bath adjusted 45° C. to 48° C. for 5 minutes.
3. Aseptically pour the cooled medium into a sterile Petri dish, tilt dish to cause medium to cover the entire bottom of the dish.
4. Allow medium to stand undisturbed until completely solidified, at 15 minutes.
5. With a sterile inoculating loop (or special streaking needle) dip the material to be cultured, then knock off the excess material fr the loop against the inside of the container. This will leave the inoculating-loop wire coated with a microscopically thin film of m terial.
6. Rub the loop on the surface of the solid medium near one edge of t dish, spreading the material over an area about the size of a dime
7. Starting at the spot of inoculation, streak back and forth across surface of the medium, taking care not to cut into the surface of agar. Keep the streaks as close together as possible (if they sho overlap slightly it will not matter). Continue streaking back and forth until the entire surface of the medium has been covered with streaks as shown in the accompanying diagram. The success of this cedure depends on having from 50 to 150 streaks on the surface of medium.



8. Invert the inoculated plates and incubate at the temperature speci by the instructor for the particular organisms being sought.

## Procedure B: The Loop Dilution (Poured Plate) Method

1. Obtain three tubes of liquid Nutrient Agar and keep them in a wate bath at a temperature of 45° C. to 48° C. for 5 minutes. Label th tubes 1, 2, and 3. Label three sterile Petri dishes 1, 2, and 3.
2. With a sterile loop transfer one loopful of the material to be cul to agar tube No. 1 and mix by stirring with the loop. Flame the l (Note: Return all tubes of agar to the water bath immediately aft transfers are made. A few minutes at room temperature will cause to solidify.)
3. Transfer two loopfuls of agar from tube No. 1 to tube No. 2 and mi Flame the loop.
4. Transfer three loopfuls of agar from tube No. 2 to tube No. 3 and Flame the loop.
5. Aseptically pour the agar in tube No. 1 into the dish No. 1, repla the cover immediately, and gently tilt the Petri dish until the ag has completely covered the bottom. In a similar manner pour the c tents of tubes Nos. 2 and 3, into Petri dishes Nos. 2 and 3, respe tively.




6. Allow the agar to solidify in the plates and label the culture.
7. Invert the plates and incubate at the temperature specified by the structor.

Second Day:

1. Observe the types of colonies developing on the plates from Proce A or B.
2. Select ten colonies representative of the predominating organisms the materials cultured, and encircle the colonies on the bottom o the dish with a wax crayon.
3. Describe the colonies and record the description on the Descripti Chart in the space for Agar Colonies.
4. Make transfers from the selected colonies to Nutrient Agar slants and incubate at an appropriate temperature.
5. Make Gram stains of slides prepared from the original colonies se and record morphologic features and Gram reaction of the organism the Descriptive Chart.

Discard any cultures which are not pure, and select new colonies fro to work.

The procedure for the Gram Stain is given in Exercise 2.

(Note: In this Exercise the instructor will provide several natural terials from which unknown bacteria will be isolated. Suitable materials this Exercise are soil, dung, polluted water, garbage, exudate from a wou soured milk or cream, cheese, chopped beef (hamburger), sweepings from fl etc. A series of ten unknown isolates should be made for intensive study throughout the course.)

## Exercise 2

# THE GRAM STAINING PROCEDURE

The Gram Stain is a valuable tool in the study of bacteria. From thi stain one can determine the morphology of the vegetative cell, the Gram st ing characteristics of the protoplast, and frequently the presence of endo spores, granular inclusions or vacuoles. Many modifications of the Gram s have been devised. The most widely used one, and one which is relatively proof, is the Hucker modification. For best results the Gram stain should performed on cultures at different ages. (See Manual of Methods for Pure Culture Study of Bacteria, Leaflet $IV_{46}$ -10 on Interpretation of the Gram Stain.)

**Procedure:**

1. Make a film on a clean glass slide of each culture under study. F with gentle heat of a Bunsen flame.
2. Flood smear for 1 minute with Hucker's Ammonium Oxalate-Crystal Vi Stain.
3. Wash thoroughly in water. (Tap water may be used here.)
4. Flood smear for 1 minute with Gram's Iodine Solution.
5. Wash in tap water and blot off excess water. Do not allow to beco completely dry.
6. Decolorize by dripping 95% Ethyl Alcohol over the slide until stre of color stop coming from the smear. (Usually about 30 seconds.)
7. Wash immediately in water.
8. Counterstain 10 seconds in Safranin Counterstain solution.
9. Wash thoroughly in tap water, blot, dry, and examine.
10. Record results on the Descriptive Chart in the space provided for Gram Stain. Be sure to record the age of the culture at the time smear was made!

Gram positive organisms are blue-violet, Gram negative organisms are and Gram variable organisms may show part blue-violet and part red, or be violet at one age and red at another age. Interpretation must be made cau tiously!

## Exercise 4

# DETERMINATION OF OPTIMUM GROWTH TEMPERATURE

The temperature of incubation employed in the original isolation of a ture might not be the optimum for the organism. In order to be assured tha organism will be functioning efficiently in subsequent determinations, one ascertain the optimum growth temperature rather early in the identification an unknown organism.

**Procedure: First Day.**

1. Make agar slant stroke cultures, in quadruplicate, of each unknown organism in the collection isolated in Exercise 1.
2. Incubate one culture of each unknown at each of the following tempe tures: 8° C., 20° C., 28° C., and 37° C. (If any thermophilic org isms are suspected, additional cultures could be added and incubate temperatures of 55° C. and 65° C.)

**Second Day.**

3. All cultures should be observed at the end of 1, 2, 4, and 7 days' cubation for growth.
4. The lowest temperature of incubation giving the <u>most abundant</u> growt will be considered the optimum for the organism in question.
5. Record the optimum growth temperature on the Descriptive Chart.

(In all subsequent experiments the unknown organisms should be incubat at the optimum temperature, unless otherwise specified!)

Exercise 5

# THE CATALASE TEST

The separation of genera within certain families of bacteria is depend upon the demonstration of catalase production by the organism in question. Because the catalase test is simple to perform, it is advisable to perform on all the unknown cultures in the collection.

**Procedure:**

1. Make one Nutrient Agar stroke culture of each unknown organism in t collection. (Extra cultures from Exercise 4 may be used for this Exercise.)
2. Incubate 48 hours at appropriate growth temperature.
3. Pour enough 3% Hydrogen Peroxide (ordinary U.S.P. strength $H_2O_2$) on the growth to fill the tube about one inch full.
4. Note the presence or absence of bubbles of gas arising from the sur face of the medium. This gas is the oxygen liberated when the hydr gen peroxide is decomposed by the enzyme catalase.
5. Record the liberation of gas bubbles as a positive catalase test on the Descriptive Chart.

# Exercise 6

## DETERMINATION OF RELATION TO FREE OXYGEN

Although most of the cultures selected from Exercise 1 will be aerobes, information relative to the relation of the organisms to free oxygen may be helpful later in explaining certain behavior patterns of the isolated cultu

**Procedure: First Day.**

1. Melt sufficient tubes of clear Nutrient Agar to have one for each cu ture in the collection of "unknowns" and steam at 100° C. for at lea 20 minutes.
2. Cool the medium to 45° C. to 48° C. in a water bath.
3. With a sterile, straight, inoculating wire pick a small amount of cu ture from the surface of the "stock" culture of an "unknown" and in- oculate the cooled medium by making a stab inoculation to the botton of the tube.
4. Chill the inoculated medium in cold water until solidification occu
5. Incubate at the optimum growth temperature for 48 hours.

**Second Day.**

6. Observe the cultures for the position and amount of growth in the me
7. Classify the organisms as: Obligate aerobes, obligate anaerobes, fa tative organisms, or microaerophiles and record on the Descriptive C in the space provided for Relation to Free Oxygen.

# Exercise 7

## DETERMINATION OF MOTILITY

Many species of bacteria possess flagella or other structures for locom tion. Because all species do not possess these structures, mobility can be used as a distinguishing feature in determinative bacteriology. Often it is sufficient to determine the presence or absence of motility without consider ing the number or position of flagella.

This Exercise describes three methods for determining motility.

**Procedure A: The Simple Mount.**

1. Clean a glass slide and cover glass thoroughly, and dry.
2. Put a small drop of water on the slide. With a sterile inoculating needle introduce a small amount of young (12 to 18 hour) culture su ficient to give very slight turbidity to the water.
3. Sterilize and cool the needle, and mix the bacterial suspension.
4. Carefully place the coverglass on the suspension.
5. Observe under the high dry objective. It will be necessary to redu the illumination to the smallest amount needed to make the bacteria cells visible. Any true motility seen should be recorded in the sp provided on the Descriptive Chart. Care must be taken to rule out ment caused by streaming as the preparation dries out. The absence motility should not be recorded, but rather the determination shoul be repeated using one of the following techniques.

(The Simple Mount method of determining motility has the advantage tha it is quick and will give accurate results for actively motile, large cells By this screening test the time necessary in carrying out the more tedious complex methods can be saved in case of the actively motile cultures.)

**Procedure B: The Hanging Drop Technique.**

1. Clean a coverglass thoroughly.
2. Put a small gob of vaseline on each corner of the coverglass.
3. Place a small loopful of water in the center of the coverglass, ino late it lightly with a young (12 to 18 hour) culture, and mix to pr duce a homogeneous suspension.
4. Quickly invert the coverglass and gently place over the depression a "hanging drop" slide. Do not press the coverglass down onto the slide. The vaseline will act as a buffer to help protect the cover glass from breakage.
5. Place a drop of immersion oil on the top of the mount and examine u the oil immersion objective. Note any vital motility, being carefu to distinguish vital from Brownian movement. Record positive resul on the Descriptive Chart.

(A negative result may occur if cultures are too old or for various ot reasons. Consequently, all negative results should be checked culturally.)

Procedure C: Motility Determination in Semi-Solid Agar.

1. Make a stab inoculation in a tube of Motility Test Agar Medium of a culture where doubtful or negative results were obtained on the microscopic motility determinations.
2. Incubate at the optimum temperature and observe after 8, 24, and 48 hours.
3. Motility is shown by the appearance of a diffuse zone of growth spreading from the line of inoculation. If the organism is very actively motile, the entire tube of medium may show the diffuse growth.

(All coverglasses and slides used in motility tests should be soaked in a disinfectant solution or be boiled for at least 10 minutes before washing them!!)

Exercise 8

# THE SPORE STAIN

## (Snyder Modification of Dorner Method)

The Family Bacillaceae is recognized by the presence of endospores in c which have started to "age". Normally it is not difficult to find these end spores in Gram stains. The endospores will appear as unstained central to p bodies of spherical to cylindric shape. Occasionally the number of endospor per smear may be very small, or the endospores can not be readily identifie such because of numerous unstained vacuoles in the cell.

To be certain of endospores, one should employ a procedure which will ferentially stain the endospore. There are many methods which can be used. Snyder modification of the Dorner spore stain is one of the best for studen Cultures between 2 and 7 days old give best results, as a rule.

**Procedure:**

1. Prepare smear and fix.
2. Cover smear with a piece of blotting paper, then saturate the blott paper with Ziehl's Carbol Fuchsin stain.
3. Steam the preparation with gently heat for 5 to 7 minutes. Be care that the blotting paper does not dry out. Add more stain as necess
4. Quickly decolorize with 95% Ethyl Alcohol, then wash in water.
5. Apply a small drop of saturated Aqueous Nigrosin Solution and sprea over the entire film.
6. Dry quickly without washing.

Spores will stain red, vegetative cells will be colorless, and the bac ground will be a purplish-black.

Record in the Descriptive Chart the size, shape, position, number of s and whether the sporangium is swollen. (See Exercise 9, Procedure B.)

# Exercise 9

## DETERMINATION OF SIZE OF BACTERIA

The size of the vegetative cell, sporangium, and endospore is signifi in identifying bacteria. All measurements are in microns. Before measure can be made, the microscope must be calibrated.

**Procedure A: Calibration of the Microscope.**

1. Place a linear ocular micrometer on top of the diaphragm in the oc and set the draw tube at 160 mm. If necessary adjust the diaphrag that the ocular micrometer is in perfect focus.
2. Place a stage micrometer on the stage and focus the low power obje upon the fine rulings. The rulings of the stage micrometer will a superimposed on the ocular micrometer.
3. Align the rulings so they are parallel, then adjust the stage micr so that the beginning line of the stage micrometer is coincident w the beginning line of the ocular micrometer.
4. Divide the value of the included spaces on the stage micrometer by number of divisions on the ocular micrometer. The quotient is the of one ocular space in linear measurement in terms of millimeters fractions of millimeters. Convert this value to microns.
5. Record the value of one ocular space in terms of microns in the ch below.
6. Repeat, using the high dry and oil immersion lenses. Record in th chart below.

| Number of Scope _____ Make _____ | | |
|---|---|---|
| Objective | Tube Length | Value of 1 ocular space in μ |
| Low Power (16 mm) | | |
| High Dry (4 mm) | | |
| Oil Immersion (1.9 mm) | | |

**Procedure B: Measurement of Bacteria.**

1. Place a stained smear of bacteria to be measured under the microsco Insert a linear ocular micrometer in the ocular of the microscope.
2. Using the oil immersion objective, view the cells. Note how many s of the ocular micrometer are covered by the length and breadth of t cell.
3. Compute the measurements in terms of microns.
4. Measure several typical looking cells and record the limits in size also the average dimensions. Bizarre-shaped cells normally should be measured.

## Exercise 10

# THE HEAT TEST FOR ENDOSPORES

In cases where the staining techniques fail to elicit endospores in or isms which might reasonably be spore-formers, the heat test should be perfo to prove the presence of a heat-resistant body. Bacilli larger than 0.8 mi in width should be regarded as potential spore-formers, especially if Gram positive.

**Procedure:**

1. Select an old stroke culture (at least one week old) of the organis be tested.
2. Make a suspension of the organism in about 3 ml. sterile water.
3. Immerse the tube of suspension in a water bath adjusted to 85° C. s that the level of the water in the bath is within one inch of the t of the tube.
4. Heat the suspension for 10 minutes; then chill the tube by placing cold water for 5 minutes.
5. Aseptically pour a tube of Nutrient Broth into the tube of heated s sion.
6. Incubate the culture at the optimum temperature. Growth appearing any time within a week is indicative of heat-resistant bodies (endo spores).

# Exercise 11

## THE ACID FAST STAIN

## (Ziehl-Neelsen Method)

Members of the genus Bacillus and some of the Actinomycetales possess unusual property of retaining dyes even in the presence of acid-alcohol sol tion. The characteristic is so specific for some forms that it can be used a distinguishing character.

**Procedure:**

1. Make a smear of a young (24 to 48 hour) culture and fix.
2. Flood smear with Ziehl's Carbol Fuchsin and heat slide gently until steam arises from the slide. (Be careful not to boil the dye or it will precipitate. Replace any dye lost by evaporation.) Steam for 5 minutes.
3. Wash in tap water.
4. Decolorize slide by flushing with Acid-Alcohol until smear becomes flesh pink in color.
5. Wash in tap water and leave slide flooded with water.
6. Pour on 3 to 4 drops of Loeffler's Alkaline Methylene Blue Stain an mix by tilting the slide. Allow to stain for 15 seconds.
7. Wash thoroughly in water, blot dry.
8. Examine under oil immersion objective. Acid-fast organisms will re the bright red fuchsin color. Non-acid-fast organisms lose the fuc dye and adsorb the methylene blue.
9. Record results of acid fast stain on the Descriptive Chart in the s provided.

(In this Exercise it is advisable to use a culture of Mycobacterium av or Myco. phlei as a control.)

# Exercise 12

## THE CAPSULE STAIN

An enlarged capsule is found in some species of bacteria. This may be significant morphological feature. Several methods are available for demonstrating the presence of a capsule, of which the following one has given good results routinely.

**Procedure: Anthony Stain**

1. Make films of young liquid culture of unknown organisms to be stained and dry in the air. Do not fix.
2. Flood film with Anthony's Stain for 5 to 7 minutes.
3. Wash off dye with 20% aqueous copper sulfate solution. Do not use water.
4. Blot dry, and examine under oil immersion objective.
5. Capsules ~~stain a bluish-violet~~ appear unstained and the vegetative cell stains dark to deep violet.

(A milk culture of Alkaligenes viscosus can be used as a control in this Exercise.)

## Exercise 13

## THE GRANULE STAIN

Members of the genera Corynebacterium and Lactobacillus contain metach tic granules in the cytoplasm. Occasional members of other genera may also show these granules. Hence, a specific stain for metachromatic granules ma useful in determinative work.

The following two methods have found wide favor as stains giving deper results in the hands of students.

**Procedure A: The Loeffler Methylene Blue Granule Stain.**

1. Prepare a film of each "unknown" organism in the collection and fix with gentle heat.
2. Flood smear with Loeffler's Alkaline Methylene Blue Stain for 30 se
3. Wash in water thoroughly.
4. Dry and examine under oil immersion objective.

The cells will stain light blue and granules, if present, will stain d blue to violet.

**Procedure B: The Albert's Granule Stain.**

1. Prepare and fix films of unknown organisms.
2. Flood slide with Albert's Diphtheria Stain for 5 minutes.
3. Wash quickly in water and shake excess wash-water from the slide.
4. Flood slide with Albert's Iodine Reagent for 1 minute.
5. Wash quickly in water and blot dry.

The metachromatic granules will be deep green to black in color. The remainder of the cytoplasm will be pale green to almost colorless.

(Note: In this Exercise it is advisable to stain, simultaneously with "unknown" cultures, a smear of Corynebacterium diphtheriae or a Lactobacill sp. which produces granules.)

## Exercise 14

# THE FLAGELLA STAIN

The problem of staining flagella and securing consistently satisfactor preparations is a real challenge to the worker in determinative bacteriolog There are many methods available. The selection of a technic is a personal matter. It would be preferable for the worker to try several methods and u the one which gives the best results in his own hands.

The Plimmer and Paine modification of the Casares-Gil's flagella stain given most consistent results in the author's laboratory and is presented h because of its relative simplicity and good stability of the "stock" mordan solution. For detailed hints on flagella staining the reader is referred t the Manual of Methods for Pure Culture Study of Bacteria.

**Procedure:**

1. Make a suspension of a young (12 to 18 hour) agar slant culture in 3 ml. of sterile water and place at the optimum growth temperature 10 minutes.
2. Wash slides in a non-soap-containing detergent (Dreft, Tide, Fab, employing a piece of cotton gauze for mild abrasion. Wash thorough in water and dry on a clean towel. Then polish the slide with a cl gauze moistened with 95% alcohol. After cleaning the slide do not touch it except on the edges.
3. Carefully lift one large loopful of culture from the surface of the suspension and gently place at one end of the cleaned slide. Tilt slide to allow the drop of suspension to run down the slide slowly
4. Dry without heat. Do not fix.
5. Flood slide with Casare-Gil's Flagella Stain Mordant (diluted 1 - by filtering directly onto the slide. Let stand without heating f 1 minute, or such time as the instructor indicates.
6. Wash in water.
7. Flood slide with Ziehl's Carbol Fuchsin Stain for 5 minutes.
8. Wash in water and stand slide on edge to dry. The flagella should be seen as red projecting threads coming from the deeply-stained, cells.
9. Note the number and position of the flagella on the cell and recor on the Descriptive Chart in the space provided for Flagella.

(It is recommended that a control of a known flagellated organism be at the same time.)

## Exercise 15

# DEMONSTRATION OF PLEOMORPHISM

Pleomorphism frequently occurs in cultures, especially with aging or t fer to different media. This phenomenon may give the student some consterr if he does not appreciate the extent to which an organism can change its m phology as a result of pleomorphism.

This Exercise is designed to impress upon the student the variability the shape of cells of an organism. The variability in morphology observed this Exercise should so impress the student that he will not be confused b similar observations in his future work.

**Procedure:**

1. Prepare a series of tubes containing 5 ml. Nutrient Agar medium and sterilize.
2. Divide the tubes into four lots.
3. To lot Number 1 add 1 ml. of sterile 10% Lithium Chloride solution, mix, and lay tubes in slanting position to solidify.
4. To lot Number 2 add 2 ml. of the LiCl solution and to lot Number 3 add 3 ml. of LiCl solution and treat as above.
5. Slant tubes from lot Number 4 without adding any LiCl.
6. When the media prepared above are solid inoculate one tube of each with a pure culture of Escherichia coli, and one tube of each lot w one of the bacilli or coccobacilli from the collection of "unknowns
7. Incubate at optimum growth temperatures for the organisms used.
8. After 48 hours make Gram stains of the growth from each slant, and the variability in the organisms with varying amounts of LiCl.

Exercise 16

# PRELIMINARY BIOCHEMICAL REACTIONS ON UNKNOWN CULTURES

Some bacteria can be traced to the Family or even the Genus with the m phological and cultural information already at hand from Exercises 1 throug 14. In most cases, however, a study of selected biochemical reactions must carried out before identification can be accomplished.

In this Exercise only those biochemical tests are included which are c sidered as the minimum essential to bring identification to the Family.

**Procedure: First Day.**

1. Inoculate each unknown organism into the following media:
   a. Glucose Broth in fermentation tubes
   b. Lactose Broth in fermentation tubes
   c. Litmus Milk
   d. Potassium Nitrate Broth
2. Divide a Frazier Gelatin Agar plate into 3 sectors. In each sector make a single stroke inoculation of the unknown organisms.
3. Incubate all cultures at optimum temperature for 48 hours.

**Second Day.**

4. Read the Glucose Broth fermentation tubes and Lactose Broth ferment tion tubes for acid and gas production.
5. Read Litmus Milk medium for acid reaction, alkaline reaction, acid rennin curd, peptonization of casein, gas production and reduction litmus.
6. Check the Potassium Nitrate Broth for nitrites as follows: To 5 ml of culture add 0.5 ml. Sulfanilic Acid Reagent and 0.5 ml. Dimethyl Alpha Naphthylamine Reagent. Production of a red color indicates presence of nitrites and thus indicates that nitrates were reduced.
7. Flood the surface of the Frazier Gelatin Agar plate with enough Frazier's Gelatin Developer to cover the surface. Let stand a few minutes. Gelatin is precipitated as a white opaque material in the medium. Presence of a clear zone around the growth of organisms in dicates gelatin hydrolysis (liquefaction).
8. Record all reactions and findings on the Descriptive Chart in the s provided.

# Exercise 17

## PRELIMINARY DETERMINATION OF FAMILY OF UNKNOWN CULTURES

With the information at hand concerning the morphological, cultural, ar preliminary biochemical characteristics of the unknown organisms, it should possible to identify the Family to which most cultures belong. The method o isolation has essentially restricted the group of unknowns to members of the Sub-Order Eubacteriineae. In cases where it is felt that some other Order i involved, consult the instructor and check in Bergey's Manual of Determinati Bacteriology, 6th Edition, pages 65 to 66.

The following dichotomous Key to the Sub-Order Eubacteriineae, adapted from pages 67 to 68 in the Bergey's Manual, makes use of characteristics whi the preceding Exercises should reveal, and should make possible the identifi tion of the Family to which the unknowns belong.

### Key to the Families of the Sub-Order Eubacteriineae

1. Coccoid forms .....
1. Not coccoid, but rod-shaped or spiral in form .....

2. Cells arranged in chains ..... Lactobacteriac (See Exercise

2. Cells not arranged in chains, but singly, paired, in packets of 4 or 8, or in irregular grapelike masses .....

3. Gram positive to Gram variable. Grow well on nutrient agar ..... Micrococcac (See Exercise

3. Gram negative. Grow poorly or not at all on nutrient agar; Body fluids usually needed for growth ..... Neisseriac (See Exercise

4. Rod-shaped cells .....
4. Spiral-shaped cells .....

5. Gram positive to Gram variable .....
5. Gram negative .....

6. Cells bear endospores ..... Bacillac (See Exercise

6. Cells do not form endospores .....

7. Cells motile .....
7. Cells non-motile .....

8. Litmus milk not changed ..... Bacteria (See Not

8. Litmus milk slightly acid, litmus reduced, not coagulated. Causes monocytosis in rabbits ..... Corynebacteria (See Exercise

9. Litmus milk usually acid and coagulated quickly ..... Lactobacteria
(See Exercise

9. Litmus milk if acid, rarely coagulated and then but slowly, or unchanged to alkaline .....

10. Metachromatic granules present ..... Corynebacteria
(See Exercise

10. No metachromatic granules ..... Bacteria
(See Not

11. Can grow in suitable inorganic media ..... Nitrobacteria
11. Can not grow in wholly inorganic media ..... Pseudomonada
(See Exercise

12. Can develop on inorganic media ..... Nitrobacteria
12. Can not develop on inorganic media .....

13. Do not grow well on media containing organic nitrogen. Fix nitrogen non-symbiotically ..... Azotobacteria
(See Exercise

13. Grow well on media containing organic nitrogen .....

14. Motile by polar flagella. Colonies often produce water-soluble green pigment or non-soluble yellow pigment ..... Pseudomonada
(See Exercise

14. Motile by peritrichous flagella or non-motile .....

15. Colonies produce pigment .....
15. Colonies colorless .....

16. Pigment violet ..... Rhizobia
(See Exercise

16. Pigment not violet .....

17. Ferment glucose with acid and gas. Litmus milk usually acid and curdled. Pigment red, orange, or yellow ..... Enterobacteria
(See Exercise

17. Ferments glucose feebly. No gas formed from sugars. Litmus milk rarely acid or curdled. Pigment orange to yellow ..... Achromobacteria
(See Exercise

18. Glucose required for growth ..... Rhizobia
(See Exercise

18. Glucose not required for growth .....

19. Grow well on nutrient agar .....
19. Grow poorly or not at all on nutrient agar. Very small bacilli ..... Parvobacteria
(See Exercise

20. Ferment glucose strongly with acid or acid and gas. Reduce nitrates. Litmus milk often curdled. ..... Enterobacteria
(See Exercise

20. Ferments glucose feebly or not at all. Litmus milk with little or no acid. Never curdled. Action on nitrates variable ..... Achromobacteria
(See Exercise

20. Organisms which fail to fall in some other Family. ..... Bacteria
(See Not

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*The placing of organisms in the Family Bacteriaceae generally should be do
with reluctance, inasmuch as the relationship of the Family to the others
uncertain, and it has assumed the character of being a "waste-basket" Fami
to contain incompletely studied organisms.

Confirmation should be made of the placement of the unknowns by check
in pages 67 to 68 in Bergey's Manual, 6th Edition.

## Exercise 18

## STUDY OF THE FAMILY BACILLACEAE

Inasmuch as the main characteristics of the genera Bacillus and Clostr are similar, only the genus Bacillus will be studied in detail. Previous E cises have given information pertaining to the morphological features of th known bacteria in the collection. For this Exercise all endospore-forming bacilli will be selected from the collection. The instructor will supply b cultures of two typical strains of the genus Bacillus, labelled with code n bers. The student will proceed to identify all the Bacillus strains to the species.

To conserve the student's time and save on supplies, it will be assume that the cultures distributed by the instructor will possess the characteri of the Genus Bacillus as cited on page 704 to 705 in Bergey's Manual.

The method of original isolation has essentially excluded thermophiles the "unknown" collection, hence temperatures of 30° C. to 37° C. will usual be satisfactory for growing the organisms.

**Procedure: First Day.**

1. Streak the broth cultures supplied by the instructor onto Nutrient A plates, using Procedure A, Exercise 1.
2. Incubate the streaked plates at 30° C. to 37° C. for 24 to 48 hours
3. Make a simple stain of the original broth culture, using Hucker's Gentian Violet Stain, to determine the morphology of the organisms. (This step is valuable in giving the student an idea of the relative size and shape of the strains he has received. Later he should chec to see whether the isolations which he makes conform in appearance t the type on this slide.)

**Second Day.**

4. After incubation of the streaked plates observe the colonies for uni ity of colony characteristics and record description on the Descript Chart.
5. Make an isolation from a typical colony, preferably in the "S" phase onto Nutrient Agar slant.
6. Make a Gram stain of the organisms in the colony selected. Record o the Descriptive Chart.
7. Incubate the slant culture at 30° C. to 37° C. for 24 hours.

**Third Day.**

8. Check the agar slant culture for purity, describe the growth, make a Gram stain, and record the Gram reaction.
9. Inoculate the following media from the slant culture and from the "s cultures" in the collection of "unknowns":

a. Motility Test Agar (stabs)*

b. Litmus Milk

c. Glucose Broth in fermentation tubes

d. Potassium Nitrate Broth

e. Citrate Agar for Bacilli (slants)

f. Ammonium Phosphate Arabinose Agar (slants)

g. Ammonium Phosphate Xylose Agar (slants)

h. Frazier's Gelatin Agar (plates)

i. Milk-Agar (plates)

j. Starch Agar (plates)

k. Acetyl Methyl Carbinol Medium for Bacilli

l. Glucose Broth for Bacilli

10. Incubate all cultures at 30° C. to 37° C. (or optimum temperature).

11. Make measurements of cell size and spore size, and record.

12. Check culture for motility by microscopic method.

(*Where the test has already been performed in Exercise 16, it is not necess to repeat it here.)

**Fourth and Subsequent Days.**

13. Observe Motility Test Agar for evidence of motility of the organism. (See Exercise 7, Procedure C.)

14. Observe Litmus Milk for the various reactions. Keep the cultures fo future readings at 2, 5, 7, and 14 days. (See Exercise 16, paragrap 5.)

15. Check the Glucose Broth in fermentation tubes for gas production.

16. Test the Potassium Nitrate Broth for the presence of nitrites as fol To 5 ml. culture, add 0.5 ml. of 0.4% KI solution and 0.5 ml. of 1% Boiled Potato Starch Paste. Mix. Add 2 drops concentrated $H_2SO_4$. to blackish color indicates the presence of nitrites.
    It is advisable to check for nitrites at 3 and 5 days' incubati

17. Check Citrate Agar slants for growth and change of reaction to alkal Growth with alkaline reaction indicates utilization of the citrate radical.

18. Observe the Ammonium Phosphate Arabinose and Xylose Agar slants for growth and acid reaction at 3, 7, 14, and 21 days. Record growth an acid reaction as positive.

19. As soon as the Frazier Gelatin Agar plates show growth pour on enoug Frazier Gelatin Developer to cover the agar medium. (See Exercise 1 paragraph 7.)

20. Observe Milk-Agar plates for hydrolysis of casein.

21. Check Starch Agar for hydrolysis of starch by pouring on enough Gra Iodine Solution to cover the medium. A colorless zone around the g indicates starch hydrolysis.

22. Acetyl Methyl Carbinol Medium should be checked as follows: To 1 m culture, add 0.6 ml. 5% Alcoholic Alpha Naphthol Solution and mix. add 0.4 ml. 40% aqueous KOH. Mix and let stand undisturbed for 30 minutes. A red layer formed at the top which progresses downward i the tube indicates presence of acetyl methyl carbinol and is record as a positive test. The culture should be checked at 2, 4, 6, 10 a 20 days, if previous tests are negative.

23. The Glucose Broth for Bacilli is held for 7 days, then a colorimetr determination of pH is made. Add 3 drops of Phenol Red Indicator t 5 ml. of culture. Check against a standard set of colorimeter tube A pH of 8.0 or above is a positive test.

24. Record all reactions and observations on the Descriptive Chart.

25. Trace each unknown organism to the species, using the key on pages to 708 in Bergey's Manual, 6th Edition, or use the key on pages 38 39 in "Aerobic Mesophilic Sporeforming Bacteria" by Smith, Gordon, Clark, U.S.D.A. Misc. Publ. #559 (1946). Then check each organism against the complete description of the species in pages 708 to 762 Bergey's Manual, 6th Edition.

## Exercise 19

# STUDY OF THE FAMILY MICROCOCCACEAE

The Family Micrococcaceae is composed of three genera: Micrococcus, cocci in irregular bunches; Gaffkya, cocci in tetrads in animal exudates or as pairs or irregular masses in ordinary media; and Sarcina, cocci in regular packets of 8 cells. Many species produce chromogenic colonies with shades of red, orange or yellow, as well as white.

For this Exercise select all cultures in the collection of "unknowns" which have the characteristics listed for Micrococcaceae on page 235 in Bergey's Manual, 6th Edition. The instructor will hand out two broth cultures of typical species of Micrococcaceae. The student should identify all strains as to the species.

**Procedure: First Day.**

1. Streak the broth cultures supplied by the instructor onto Nutrient Agar plates, using Procedure A, Exercise 1.
2. Incubate the streaked plates at 20° C. to 37° C. as indicated by the instructor.
3. Make a simple stain of the original broth culture, using Hucker's Gentian Violet Stain, to determine the morphology of the organisms. (This stain is valuable in giving the student an idea of the relative size and shape of the strains he has received. Later he should check to see whether the isolations which he makes conform in appearance to the type on the slide.)

**Second Day.**

4. After incubation of the streaked plates observe the colonies for uniformity of colony characteristics and record the description on the Descriptive Chart.
5. Make an isolation from a typical colony preferably in the "S" phase onto Nutrient Agar slant.
6. Make a Gram stain of the organisms in the colony selected. Record on the Descriptive Chart.
7. Incubate the slant culture at 20° C. to 37° C. as indicated by the instructor.

**Third Day.**

8. Check the Nutrient Agar slant culture for purity, describe growth on the Descriptive Chart, and make a Gram Stain and record the Gram reaction. Note any chromogenesis.
9. Inoculate the following media from the Nutrient Agar slant cultures from the "stock cultures" of Micrococcaceae in the collection of "unknowns":

a. Potassium Nitrate Broth*

b. Litmus Milk

c. Frazier Gelatin Agar (plates).

d. Ammonium Phosphate Glucose Agar (slants).

e. Urea Glucose Agar (slants).

f. Motility Test Agar (stabs)

g. Mannitol B C P Agar (stabs)

h. Glucose B C P Agar (stabs)

i. Lactose B C P Agar (stabs)

j. Sorbitol B C P Agar (stabs)

k. Glycerol B C P Agar (stabs)

l. Glucose Broth in fermentation tubes

(*Where the test has already been performed in Exercise 16 it is not necessa to repeat it here.)

10. Incubate all cultures at the appropriate temperature for 48 hours.

11. Make measurements of cell size, and record on the Descriptive Chart. Also record the orientation of the cells.

12. Check the culture for motility microscopically. (See Exercise 7, Procedure A or B.)

**Fourth and Subsequent Days.**

13. Test the Potassium Nitrate Broth for nitrites. (See Exercise 16, paragraph 6.)

14. Check the various reactions in Litmus Milk. (See Exercise 16, paragr 5.)

15. Check the Frazier Gelatin Agar plates for hydrolysis of gelatin. (See Exercise 16, paragraph 7.)

16. Observe the Ammonium Phosphate Glucose Agar slants and Urea Glucose A slants for growth and acid production.

17. Check Motility Test Agar stabs for motility. (See Exercise 7, Proced C, paragraph 3.)

18. Observe for acid production in the Mannitol, Glucose, Lactose, Sorbit and Glycerol B C P Agar stabs. A change to a yellow color by the Bro Cresol Purple indicator indicates acid production.

19. Observe the Glucose Broth fermentation tubes for presence of acid and gas.

20. Record all observations and reactions in the space provided in the Descriptive Chart.

21. Trace each unknown organism to the Genus and species, using the keys on pages 235 to 237, 283, and 285 in Bergey's Manual, 6th Edition. Then check each organism against the complete description of the spec as listed in pages 235 to 294 in Bergey's Manual, 6th Edition.

## Exercise 20

## STUDY OF THE FAMILY LACTOBACTERIACEAE

The Family Lactobacteriaceae contains seven genera in two tribes. Th entire group is highly fermentative. Most of the species do not reduce nitrates. Because of the aerobic method for original isolation of the "unknowns", the collection probably is devoid of Butyribacterium and Propi bacterium species.

The student will be given three cultures of typical, aerobic or micro aerophilic members of this Family. These should be identified as to the s along with all members in this Family from the "unknown" collection.

**Procedure: First Day.**

1. Make a suspension of each unknown organism in about 3 ml. of steri distilled water, unless the organism is already in a liquid culture
2. Streak each organism on the following media:
   a. Yeast Extract Glucose Agar (plates)
   b. Blood Agar (plates)
   c. Nutrient Agar (plates)
3. Place all plates inverted in a desiccator or $CO_2$ jar. Put a small piece of candle on top of the pile of plates. Light the candle and replace the lid gently. Allow the expanding gases to escape; then when the flame begins to die down, seal the lid securely onto the vessel.
4. Incubate the cultures at the temperature specified by the instructo for 48 hours.
5. Make a simple stain of the organisms supplied for a preliminary stu of morphology.

**Second Day.**

6. Remove the plates from the $CO_2$ jar and note upon which medium each organism grew best. Record in the Descriptive Chart.
7. Describe the colonies on the medium showing best growth. Note any hemolysis of the Blood in the Blood Agar medium. Record on the Descriptive Chart.
8. Make Gram stains of the colonies, and note morphology and Gram reac
9. Inoculate the following media from well isolated pure colonies of e organism:
   a. Litmus Milk
   b. Potassium Nitrate Broth
   c. Yeast Extract Glucose Agar (stab)
   d. Starch Agar (plate)
   e. Starch Broth in fermentation tube
   f. Blood Agar (slant)

10. Incubate for 48 hours at the temperature specified by the instructo

**Third Day.**

11. Observe the Litmus Milk culture for the various reactions. (See Exercise 16, paragraph 5.) Keep the tube for future observations.
12. Check the Potassium Nitrate Broth for growth. Remove a part of the culture to another test tube. On one portion perform the test for nitrites. (See Exercise 16, paragraph 6 or Exercise 18, paragraph
13. Observe the Yeast Extract Glucose Agar stabs for growth, and determ the oxygen relationship of the organisms (See Exercise 6).
14. Perform the test for starch hydrolysis on the Starch Agar plates (See Exercise 18, paragraph 21).
15. Observe for acid production in the Starch Broth Fermentation tube.
16. Using the key on page 305 in the 6th Edition of Bergey's Manual, classify each organism to the Tribe, and where possible also to the Genus.
17. Inoculate all Streptococceae isolates from the Blood Agar slant int the following media:
    a. 6.5% Sodium Chloride Agar (stabs)
    b. pH 9.6 Nutrient Agar (stabs)
    c. 0.1% Methylene Blue Milk Medium
    d. Sodium Hippurate Broth
    e. Peptone Yeast Extract Broth
    f. Frazier Gelatin Agar (plate)
    g. Arabinose Broth (fermentation tube)
    h. Xylose Broth (fermentation tube)
    i. Glucose Broth (fermentation tube)
    j. Lactose Broth (fermentation tube)
    k. Sucrose Broth (fermentation tube)
    l. Maltose Broth (fermentation tube)
    m. Trehalose Broth (fermentation tube)
    n. Raffinose Broth (fermentation tube)
    o. Inulin Broth (fermentation tube)
    p. Dextrin Broth (fermentation tube)
    q. Glycerol Broth (fermentation tube)
    r. Mannitol Broth (fermentation tube)
    s. Sorbitol Broth (fermentation tube)
    t. Salicin Broth (fermentation tube)
    u. Esculin Broth (fermentation tube)

18. Inoculate all cultures of the Lactobacilleae from the Yeast Extrac Glucose Agar stab into the following media:

    a. Arabinose Broth (fermentation tubes)

    b. Rhamnose Broth (fermentation tube)

    c. Xylose Broth (fermentation tube)

    d. Glucose Broth (fermentation tube)

    e. Lactose Broth (fermentation tube)

    f. Sucrose Broth (fermentation tube)

    g. Maltose Broth (fermentation tube)

    h. Raffinose Broth (fermentation tube)

    i. Dextrin Broth (fermentation tube)

    j. Mannitol Broth (fermentation tube)

    k. Sorbitol Broth (fermentation tube)

19. Incubate all cultures inoculated in paragraphs 17 and 18 above at t temperature specified by the instructor.

**Fourth Day and Subsequent Days.**

20. Observe the 6.5% Sodium Chloride Agar stabs and pH 9.6 Nutrient Aga stabs for growth.

21. Check the 0.1% Methylene Blue Milk medium for curd formation and/or reduction of the methylene blue dye. Either reaction is indicative of growth.

22. Check the Sodium Hippurate Broth for hippurate hydrolysis as follow To 0.8 ml. of culture add 0.2 ml. of Ferric Chloride Reagent. Mix. Observe after 15 minutes. A permanent precipitate indicates presen of benzoic acid (a positive test for hippurate hydrolysis).

23. Test the Peptone Yeast Extract Broth for ammonia as follows: To 5 of culture add 0.2 ml. of Nessler's Reagent. Also run a control or uninoculated tube of Peptone Yeast Extract Broth. A yellow color i the culture deeper in intensity than the control tube indicates amm production.

24. Test the Frazier Gelatin Agar plate for gelatin hydrolysis. (See Exercise 16, paragraph 7.)

25. Observe all fermentation media for presence of acid. A color chang the Brom Thymol Blue indicator to yellow indicates acid production.

26. Record all reactions and observations on the Descriptive Chart.

27. Using the 6th Edition of Bergey's Manual pages 305, 306, 313 to 315 346, 349, 350, 370, 372 to 373, and 380, identify the species of ea unknown organism. The following Key to the Lactobacillus species i based on characteristics and tests revealed in this Exercise and ma be helpful. In any case, the tentative identification must be chec against the Bergey's Manual, 6th Edition, pages 305 to 380.

## Key to the Species of the Genus *Lactobacillus*

1. Gas formed from carbohydrates .....
1. Gas not formed from carbohydrates .....

    2. Acid formed from sucrose .....
    2. No acid formed from sucrose .....

3. Acid formed from lactose .....
3. No acid formed from lactose .....

    4. Optimum temperature 45° C. to 62° C. ..... L. thermoph
    4. Optimum temperature below 45° C. .....

5. Optimum temperature 37° C. to 40° C. .....
5. Optimum temperature 28° C. to 32° C. .....

    6. Microaerophilic ..... L. acidoph
    or L. caucas
    or L. la
    6. Anaerobic ..... L. bif

7. Ferments sucrose and maltose as readily as lactose ..... L. plant
7. Ferments lactose more readily than sucrose or maltose ..... L. c

    8. Optimum temperature 37° C. ..... L. leichma
    8. Optimum temperature 45° C. ..... L. delbrue

9. Acid formed from maltose ..... L. helvet
9. No acid formed from maltose ..... L. bulgar

    10. Acid formed from sucrose .....
    10. No acid formed from sucrose ..... L. br

11. Acid formed from arabinose .....
11. No acid formed from arabinose ..... L. ferm

    12. Acid formed from xylose ..... L. buch
    12. No acid formed from xylose ..... L. pastori

## Exercise 21

# STUDY OF THE FAMILY PSEUDOMONADACEAE

The Family Pseudomonadaceae is composed of Gram negative bacilli or sp possessing polar flagella. Members of the Genus Xanthomonas form yellow, w insoluble pigments. Many members of the Genus Pseudomonas produce water-so yellow to blue-green pigments. Because of the special metabolism of Methan monas, Acetobacter, Protaminobacter species and the Spirilleae, these group will not be studied.

The instructor will supply a young Nutrient Broth culture of a typical member of the Family Pseudomonadaceae. The student will also select from h "unknown" collection all cultures belonging to this Family, and proceed to identify the species of all the Pseudomonadaceae members.

**Procedure: First Day.**

1. Streak the broth cultures supplied by the instructor onto Nutrient plates, using Procedure A, Exercise 1.
2. Incubate the streaked plates at 30° C. for 24 to 48 hours.
3. Make a Gram stain of the original Nutrient Broth culture supplied b the instructor to determine tentatively the morphology of the unkno

**Second Day.**

4. After the incubation of the streaked plates, observe the colonies f uniformity of colony characteristics and chromogenesis. Describe c the Descriptive Chart.
5. Make an isolation from a typical colony, preferably in the "S" phas onto a Nutrient Agar slant.
6. Make a Gram stain of the colony selected. Record Gram reaction.
7. Incubate the Nutrient Agar slant culture at 30° C. for 24 to 48 hou

**Third Day.**

8. Check the Nutrient Agar slant culture for purity and describe growt on the Descriptive Chart; make a Gram stain and record the Gram rea tion. Note any chromogenesis!
9. Inoculate all the cultures into the following media:
   a. Motility Test Agar (stabs)
   b. Frazier Gelatin Agar (plate)
   c. Litmus Milk
   d. 5% Sodium Chloride Agar (slant)
   e. Glycerol Agar for Pigment Production (slant)
   f. Starch Agar (plate)
   g. Glucose Broth (fermentation tube)
   h. Sucrose Broth (fermentation tube)
   i. Potassium Nitrate Broth (fermentation tube)

10. Check for motility microscopically. (See Exercise 7, Procedure A

11. Incubate all cultures at 30° C., or optimum temperature, for 24 t hours.

**Fourth Day and Subsequent Days.**

12. Check Motility Test Agar medium for evidence of motility. (See E cise 7, Procedure C.)

13. Observe the Frazier Gelatin Agar plate for chromogenesis, then te for gelatin hydrolysis. (See Exercise 16, paragraph 7.)

14. Read the Litmus Milk for all changes. (See Exercise 16, paragrap

15. Observe the 5% Sodium Chloride Agar slant for growth. If the org ism is not affected by the high sodium chloride level, it will gr luxuriantly.

16. Check the Glycerol Agar for Pigment Production for the presence o water-soluble yellow or blue-green pigment.

17. Test the Starch Agar plate for starch hydrolysis. (See Exercise paragraph 21.)

18. Observe the Glucose and Sucrose fermentation tubes for acid and g production.

19. Note any gas in the Potassium Nitrate fermentation tube. Record. move about 5 ml. of the culture and test the medium for the prese of nitrites. (See Exercise 16, paragraph 6 or Exercise 18, parag 16.)

20. Record all observations and tests on the Descriptive Chart.

21. Using the Keys on pages 82, 83 to 88, 150 to 152, and 191 in Berge Manual, 6th Edition, identify the species of the organisms. Check the tentative identification by consulting the detailed descriptio in the Bergey's Manual, 6th Edition.

# Exercise 22

## STUDY OF THE FAMILY ACHROMOBACTERIACEAE

The Family Achromobacteriaceae is somewhat closely related morphologi and physiologically to the Enterobacteriaceae. As a result, there may be overlapping of biochemical characteristics of the two groups. Generally, Achromobacteriaceae can be recognized by their relatively weak fermentativ powers and inability to produce gas from carbohydrates. Motile forms exhi peritrichous flagellation.

The instructor will furnish pure cultures of typical members of the F Achromobacteriaceae as 24 hour Nutrient Broth cultures. The student will identify all members of this Family which he has in his original collectio "unknowns".

**Procedure: First Day.**

1. Streak the Nutrient Broth cultures supplied by the instructor onto Nutrient Agar plates, using Procedure A, Exercise 1.
2. Incubate the streaked plates at 30° C. for 24 to 48 hours.
3. Make a Gram stain of the original Nutrient Broth culture supplied the instructor to determine tentatively the morphology of the unkn

**Second Day.**

4. After incubation observe the colonies on the streaked plates for uniformity of colony characteristics and chromogenesis. Describe the Descriptive Chart.
5. Make an isolation from a typical colony, preferably in the "S" pha onto a Nutrient Agar slant.
6. Make a Gram stain of the colony selected. Record Gram reaction.
7. Incubate the Nutrient Agar slant culture at 30° C. for 24 to 48 ho

**Third Day.**

8. Check the Nutrient Agar slant culture for purity, describe growth the Descriptive Chart, make a Gram stain and record the Gram react Note any chromogenesis!
9. Inoculate all cultures into the following media:
    a. Motility Test Agar (stabs)
    b. Frazier Gelatin Agar (plates)
    c. Starch Agar (plates)
    d. Peptone Iron Agar (stabs)
    e. Potato Slants
    f. Litmus Milk
    g. Potassium Nitrate Broth
    h. Glucose Broth (fermentation tubes)
    i. Sucrose Broth (fermentation tubes)

10. Incubate all cultures at 30° C., or optimum temperature, for 48 h

**Fourth Day and Subsequent Days.**

11. Check Motility Test Agar stabs for evidence of motility. (See Ex cise 7, Procedure C, paragraph 3.)
12. Test the Frazier Gelatin Agar for gelatin hydrolysis. (See Exerc paragraph 7.)
13. Test the Starch Agar for starch hydrolysis. (See Exercise 18, paragraph 21.)
14. Observe the Peptone Iron Agar stabs for hydrogen sulphide produc A blackening along the line of inoculation indicates hydrogen su production, and is recorded as a positive test.
15. Observe and describe the growth on the Potato Slants. Note any chromogenesis in the growth, also any change in the color of the Potato Slant itself.
16. Observe the various changes in the Litmus Milk. (See Exercise 1 paragraph 5.) Also note any ropiness of the milk.
17. Test the Potassium Nitrate Broth for the presence of nitrites. Exercise 16, paragraph 6 or Exercise 18, paragraph 16.)
18. Observe the Glucose, Sucrose, and Lactose Broth fermentation tube acid and gas production. Members of this Family do not produce If gas production occurs, hold the cultures for study in Exercis
19. Record all observations and tests on the Descriptive Chart.
20. Using the Keys in Bergey's Manual, 6th Edition, on pages 412, 41 417, 427 to 428 identify the unknowns to the species. Check all tative identification by comparison with the detailed descriptio in pages 412 to 439 in the Bergey's Manual, 6th Edition.

## Exercise 23

# STUDY OF THE FAMILY ENTEROBACTERIACEAE

The Family Enterobacteriaceae is composed of nine genera. Each Genus a reasonably clear-cut set of biochemical reactions. Consequently, the gr is well adapted for study in courses in bacterial identification. The wid spread contact with members of this Family in diagnostic, food, sanitary, and dairy bacteriology almost demands a thorough knowledge of all the gene in the Family. The outstanding characteristic of these small, Gram-negati bacilli is their highly fermentative nature.

Because some members of the Enterobacteriaceae are highly pathogenic, utmost precautions must be taken to see that the laboratory does not becom contaminated or the personnel infected.

Ordinarily the species of Salmonella are identified by serologic typi procedures. Classes in Serology usually consider the antigenic structure bacteria and use selected Salmonella species for test material. To avoid duplication of work in Serology courses the Salmonella species will be stu culturally and biochemically even though such methods are, admittedly, inf to serologic methods.

Broth cultures of several representative species of this Family will supplied by the instructor.

**Procedure: First Day.**

1. Streak the broth cultures supplied by the instructor onto Nutrient Agar plates, using the method outlined in Exercise 1, Procedure A.
2. Incubate the streaked plates at 37° C. for 24 to 48 hours.
3. Make Gram stains of the original broth cultures supplied to determ tentatively the morphology.

**Second Day.**

4. After incubation of the streaked plates observe the colonies for u formity of colony characteristics and record the description on th Descriptive Chart. Note any chromogenesis!
5. Make an isolation from a typical colony, preferably in the "S" sta onto a Nutrient Agar slant.
6. Make a Gram stain of the organisms in the colony selected.
7. Incubate the slant cultures at 30° C. to 37° C. as instructed for 24 hours.
8. Check motility of the organisms microscopically. (See Exercise 7, Procedure A or B.) Record all results on the Descriptive Chart.

**Third Day.**

9. Check the Nutrient Agar slant cultures for purity and describe the growth; make Gram stain to check the Gram reaction and record all results on the Descriptive Chart.

10. Inoculate the following media from the Nutrient Agar slants and from "stock cultures" in the collection of "unknowns":

    a. Motility Test Agar (stabs)

    b. Glucose Broth (fermentation tubes)

    c. Lactose Broth (fermentation tubes)

    d. Urea Broth Medium

    e. M.R.-V.P. Medium

11. Make a suspension of the growth on the Nutrient Agar slant culture o each unknown organism in a small amount of sterile saline. With a s inoculating loop, transfer a loopful of the suspension to a tube of Koser's Citrate Medium.

12. Incubate all the cultures at 37° C., or optimum temperature, for 24 48 hours.

**Fourth Day.**

13. Check the Motility Test Agar stabs for evidence of motility. (See E cise 7, Procedure C, paragraph 3.)

14. Observe Glucose and Lactose fermentation tubes for acid and/or gas.

15. Observe the Urea Broth Medium for alkali production. The Phenol Red indicator turns bright red in alkaline solutions. An alkaline react indicates urea hydrolysis (a positive test).

16. Test the M.R.-V.P. Medium for Acetyl Methyl Carbinol as follows: Re 1 ml. of the culture to a Wasserman test tube. Add 0.6 ml. of 5% Al holic Alpha Naphthol Solution. Shake to mix. Add 0.4 ml. of 40% aq ous KOH solution. Mix. Let stand undisturbed for 30 minutes. A br red layer at the top of the mixture indicates the presence of Acetyl Methyl Carbinol (a positive Voges-Proskauer test).

17. To the remainder of the M.R.-V.P. Medium add 5 drops of Methyl Red I cator. A red color is a positive Methyl Red Test. A yellow color i negative test. An orange color is a questionable test, in which cas the entire test should be repeated on a new tube of M.R.-V.P. Mediu with incubation for 96 hours.

18. Record all tests and observations on the Descriptive Chart.

19. Using the Keys on pages 444, 463, 479, 492 in Bergey's Manual, 6th Edition, identify each unknown to the Genus.

**Fifth Day.**

20. Inoculate each member of the Genus Escherichia and Paracolobactrum i

    a. Peptone Iron Agar (stabs)

    b. Tryptone Broth

    c. Sucrose Broth (fermentation tubes)

    d. Salicin Broth (fermentation tubes)

    e. Frazier Gelatin Agar (plates)

21. Inoculate each member of the Genus Aerobacter into:
    a. Frazier Gelatin Agar (plates)
    b. Glycerol Broth (fermentation tubes)
22. Inoculate each member of the Genus Erwinia into:
    a. Frazier Gelatin Agar (plates)
    b. Starch Agar (plates)
    c. Potassium Nitrate Broth
    d. Peptone Iron Agar (stabs)
23. Inoculate each member of the Genus Serratia into:
    a. Frazier Gelatin Agar (plate)
    b. Nutrient Gelatin (stabs)
24. Inoculate each member of the Genus Proteus into:
    a. Peptone Iron Agar (stabs)
    b. Tryptone Broth
    c. Sucrose Broth (fermentation tubes)
    d. Maltose Broth (fermentation tubes)
    e. Mannitol Broth (fermentation tubes)
25. Inoculate each member of the Genus Salmonella into:
    a. Peptone Iron Agar (stabs)
    b. Litmus Milk
    c. Frazier Gelatin Agar (plates)
    d. Potassium Nitrate Broth
    e. Arabinose Broth (fermentation tubes)
    f. Xylose Broth (fermentation tubes)
    g. Sucrose Broth (fermentation tubes)
    h. Maltose Broth (fermentation tubes)
    i. Mannitol Broth (fermentation tubes)
    j. Inositol Broth (fermentation tubes)
    k. Phenol Red Tartrate Agar (stabs)
26. Inoculate each member of the Genus Shigella into:
    a. Litmus Milk
    b. Tryptone Broth
    c. Xylose Broth (fermentation tubes)
    d. Rhamnose Broth (fermentation tubes)
    e. Mannitol Broth (fermentation tubes)
    f. Dulcitol Broth (fermentation tubes)
27. Incubate all Erwinia and Serratia cultures at 28° C. The remain

Sixth and Subsequent Days.

28. Observe the Peptone Iron Agar stabs for $H_2S$ production. (See Exe 22, paragraph 14.)

29. Test the Tryptone Broth for Indole by the Goré test as follows: the cotton stopper from the Tryptone Broth culture and moisten th underneath side with several drops of Ehrlich's Reagent #1, and t with an equal amount of Ehrlich's Reagent #2. Replace the stoppe the tube, and push it down until within 1 inch of the medium. Pl the tube erect in a boiling water bath for 10 minutes. Be carefu medium does not touch the stopper. Formation of a red color on t stopper indicates the presence of indole. There is no color chan with a negative test.

30. Observe all fermentation tubes for acid and gas production. Test any ropiness of the medium.

31. Test the Frazier Gelatin Agar plates for gelatin hydrolysis. (Se Exercise 16, paragraph 7.)

32. Test the Starch Agar plates for starch hydrolysis. (See Exercise paragraph 21.)

33. Check the Potassium Nitrate Broth for the presence of nitrites. Exercise 16, paragraph 6 or Exercise 18, paragraph 16.)

34. Read Litmus Milk for various changes. (See Exercise 16, paragraph Also note any ropiness.)

35. Chill the Nutrient Gelatin stabs in cold water or in the refrigera for 30 minutes. Observe any liquefaction, its type, and any chro genesis.

36. Check the Phenol Red Tartrate Agar stabs for acid. A yellow colo dicates acid formation.

37. Record all observations and tests on the Descriptive Chart.

38. Using the Keys in the Bergey's Manual, 6th Edition, on pages 445, 460, 463 to 464, 479, 486, and 535 identify all the species of the bers of Escherichia, Aerobacter, Paracolobactrum, Erwinia, Serrati Proteus, and Shigella, respectively.

39. For members of the Genus Salmonella use the following Key, based o cultural characteristics of the group, to identify the species. a more accurate determination of Salmonella species or varieties, antigenic studies must be made!)

40. Check the tentative identification of the various "unknowns" by co parison with the detailed descriptions in Bergey's Manual, 6th Edi for all species except the Salmonella species. The latter Genus c be checked in the 4th or 5th Edition of Bergey's Manual.

## Key to the Species of the Genus Salmonella

1. Cells motile .....
1. Cells not motile .....

    2. Gas formed from glucose .....
    2. No gas formed from glucose ..... Sal. typ

3. Acid and gas formed from xylose .....
3. No acid or gas from xylose ..... Sal. paraty

    4. Acid and gas formed from arabinose .....
    4. No acid or gas formed from arabinose .....

5. Hydrogen sulphide formed .....
5. Hydrogen sulphide not formed ..... Sal. typhis

    6. Acid and gas formed from inositol .....
    6. No acid or gas formed from inositol ..... Sal. hirschfe
    or Sal. enteriti

7. Tartrates fermented with acid production ..... Sal. typhimur
    or Sal. morbifi
    or Sal. ana
7. Tartrates not fermented ..... Sal. schottmuell

    8. Hydrogen sulphide formed ..... Sal. choleraesuis
    Kunzend
    8. Hydrogen sulphide not formed ..... Sal. choleraes

9. Gas formed from glucose ..... Sal. pull
9. Acid only formed from glucose .....

    10. Acid formed from mannitol, xylose, dulcitol, and dextrin .....
    10. Acid not produced from mannitol, xylose, dulcitol or dextrin ..... Shigella
    (See Bergey Manu

11. Lactose fermented with slow acid production ..... Shigella
    (See Bergey Manu
11. No acid from lactose ..... Sal. gallina

## Exercise 24

# STUDY OF THE FAMILY PARVOBACTERIACEAE

The Family Parvobacteriaceae is characterized by being composed of v small, Gram-negative rods, many of which require body fluids for growth. identification of genera and species in this Family is based on cultural biochemical characteristics which have been previously performed in the s of other Families. Nothing new would be added to the student's knowledge repeat these tests on this group. Pathogenicity tests are required for definite identification of other species. These may be difficult or dang ous to perform except in specially equipped laboratories. Because of the highly pathogenic nature of the members of this Family, only the organism Pasteurella multocida will be studied morphologically.

**Procedure: First Day.**

1. Streak the culture supplied onto a Blood Agar plate.
2. Incubate in an atmosphere of 10% $CO_2$ at 37° C.

**Second Day.**

3. Make a Gram stain of a colony. Note the appearance of the cells their Gram reaction.
4. Measure the cells. Compare to the size of the members of the Ach bacteriaceae and Enterobacteriaceae. The small size of the cells characteristic of the Family Parvobacteriaceae.
5. Make a smear and stain 15 sec. with Loeffler's Alkaline Methylene Stain diluted 1 to 3. Observe under oil immersion for bipolar st cells. Bipolar staining of the cells is characteristic of some m bers of this Family. (See Bergey's Manual, 6th Edition, pages 54 595.)

## Exercise 25

# STUDY OF THE FAMILY CORYNEBACTERIACEAE

The Family Corynebacteriaceae is recognized primarily by the characte istic, Gram-positive, club-shaped to elongate cells which frequently conta metachromatic granules. The so-called "snapping" division of the cells fr quently produces a V-shaped arrangement of the cells.

The identification of species is based largely on pathogenicity and h specificity. The cultural and biochemical tests employed in identificatio studies are those previously studied. The Genus Corynebacterium is the la est in the Family and is selected for study morphologically.

**Procedure: First Day.**

1. Streak the broth culture of a species of Corynebacterium supplied the instructor onto a Blood Agar plate.
2. Incubate at 37° C. for 24 to 48 hours.

**Second Day.**

3. Make a Gram stain and note the Gram reaction and shape and size of the cells.
4. Make a Granule Stain. (See Exercise 13.) Note the presence of me chromatic granules, and the shape and arrangement of the cells.

## Exercise 26

# STUDY OF THE FAMILY RHIZOBIACEAE

The Family Rhizobiaceae has three genera, two of which are more freque encountered than the other, namely, Rhizobium and Chromobacterium. The for is the genus to which the symbiotic nitrogen-fixing organisms belong. It w not ordinarily be isolated from the source materials listed in the fore par this Manual, hence is not considered in detail. The genus Chromobacterium, however, may be found in many natural source materials hence will be studie here in detail.

The student will identify all members of the genus Chromobacterium he have in his collection along with the culture supplied by the instructor.

**Procedure: First Day.**

1. Streak the "unknowns" upon Glucose Agar plates.
2. Incubate at 30° C. for 48 hours.
3. Make a Gram stain of the original culture supplied by the instructo and note the shape, relative size of the cells, and the Gram reacti

**Second Day.**

4. Observe colonies for pigment production.
5. Make two Glucose Agar slant cultures from a typical colony.
6. Incubate one agar slant at 37° C. and the other at 30° C.
7. Make Gram stains of the organisms from the colony selected. Note t morphology and Gram reaction. Record all results on the Descriptiv Chart.

**Third Day.**

8. Check Nutrient Agar slants for purity and describe; make Gram stain note Gram reaction. Note optimum growth temperature.
9. Inoculate the following media from the Nutrient Agar slant culture the organisms supplied and from the "stock" cultures in the collecti of "unknowns":
    a. Glucose Broth (fermentation tubes)
    b. Maltose Broth (fermentation tubes)
    c. Sucrose Broth (fermentation tubes)
    d. Potassium Nitrate Broth
10. Incubate the cultures from paragraph 9 at the optimum temperature f 48 hours.
11. Make a Flagella Stain. (See Exercise 14.) Note the type of flagell tion.

Fourth Day.

12. Observe the Glucose, Maltose, and Sucrose Broth fermentation tubes f acid production.

13. Test the Potassium Nitrate Broth for nitrites. (See Exercise 16, pa graph 6 or Exercise 18, paragraph 16.)

14. Record all observations and tests on the Descriptive Chart.

15. Using the Key on page 231, Bergey's Manual, 6th Edition, identify th members of the Chromobacterium. Check the tentative identification against the detailed description in pages 231 to 234 in Bergey's Man 6th Edition.

## Exercise 27

# STUDY OF THE FAMILY AZOTOBACTERIACEAE

The Family Azotobacteriaceae is comprised of only one Genus, Azotobact This Genus has the unique ability to grow on a suitable nitrogen-free mediu and metabolize free atmospheric nitrogen, if supplied with available carboh drates or other energy sources. The cells are much larger than most forms studied so far. The instructor will supply alkaline soils from which the student will isolate a culture of Azotobacter sp. for morphological study.

**Procedure: First Day.**

1. Pour a plate of Nitrogen-Free Mannitol Agar and allow to solidify.
2. Place a small amount of the soil sample into a small seive, and sha the seive gently over the plate to inoculate the agar with an even, light sprinkling of soil particles.
3. Invert the plate and incubate at 25° C. for 2 to 7 days.

**Second Day.**

4. Observe the plate for the presence of large, mucoid colonies surrou ing the soil particles. The colonies may be colorless or show a br pigmentation.
5. Pick off a small amount of a characteristic colony and suspend in 3 of sterile water or saline.
6. Streak the suspension onto a new Nitrogen-Free Mannitol Agar plate, order to secure well isolated pure colonies.
7. Incubate the streaked plate at 25° C. for 2 to 7 days.

**Third Day.**

8. Observe the plate culture for typical, mucoid colonies. Note any p ment production.
9. Make a Gram stain of the growth. Note the size, shape, arrangement Gram reaction of the cells.
10. Measure the cell size.
11. Perform a capsule stain on the organism. (See Exercise 12.)

## Exercise 28

## STUDY OF THE FAMILY NEISSERIACEAE

The Family Neisseriaceae is composed of Gram-negative cocci which gro best on various media enriched with mammalian body fluids. This group may encountered in cultures made from the mucous membranes of the body, hence, their inclusion in this Manual. All cultures of this Family in the studen collection and the unknown supplied by the instructor are to be identified the species.

**Procedure: First Day.**

1. Streak the broth culture supplied by the instructor onto a Chocola Agar plate, using the method outline in Exercise 1, Procedure A.
2. Place the plate inverted in a desiccator or $CO_2$ jar. Put a small p of candle on top of the pile of plates. Light the candle and repla the lid gently. Allow the expanding gases to escape; then when the flame begins to die down, seal the lid securely onto the vessel.
3. Incubate at 37° C. for 48 hours.
4. Make a simple stain of the culture supplied for a preliminary study morphology.

**Second Day.**

5. Remove the plates from the $CO_2$ jar and observe the colonies. Note appearance of the colonies and record on the Descriptive Chart.
6. Make a Gram stain of the organisms in a typical colony and record t morphology, cell size, and Gram reaction.
7. Streak two Chocolate Agar slants with the growth of the colony used for the Gram stain.
8. Incubate one slant culture at 37° C. and one at 20° C. under a 10% $CO_2$ atmosphere.
9. On several typical colonies add a few drops of fresh 1% Aqueous Par Aminodimethylaniline Monohydrochloride (Di Methyl Para Phenylene Diamine Hydrochloride). Note any change in the color of the coloni A change from pink to maroon to black identifies oxidase positive colonies. Record the oxidase test results on the Descriptive Chart the bottom margin.

**Third Day.**

10. Check the growth on the Chocolate Agar slants for purity. Note any chromogenesis. Also note the optimum growth temperature.
11. Inoculate the following media:
    a. Proteose No. 3 Agar (stabs)
    b. Glucose Broth (fermentation tubes)
    c. Fructose Broth (fermentation tubes)
    d. Maltose Broth (fermentation tubes)

e. Sucrose Broth (fermentation tubes)

f. Mannitol Broth (fermentation tubes)

g. Peptone Water Medium in fermentation tube

h. Loeffler's Blood Serum (slants)

12. Incubate the cultures under 10% $CO_2$ at optimum temperature.

**Fourth and Subsequent Days.**

13. Observe the Proteose No. 3 Agar stabs for the relation of organism free oxygen.

14. Check the Glucose, Fructose, Maltose, Sucrose, and Mannitol Broth fermentation tubes for acid production.

15. Note formation of gas in the Peptone Water Medium.

16. Note chromogenesis on the Loeffler's Blood Serum slants.

17. Record all observations on the Descriptive Chart.

18. Using the Keys on pages 295 to 296 and 302 in Bergey's Manual, 6th Edition, identify the organisms to the species.

19. Confirm the tentative identification by checking against the detail descriptions of the species on pages 295 to 304 in the Bergey's Man 6th Edition.

## Appendix I

# A COMPILATION OF MEDIA EMPLOYED IN THE EXERCISES IN THE MANUAL

For the benefit of those who may be required to make their own media, the lowing collection of media used in the preceeding Exercises has been com- ed. An asterisk (*) is placed after the names of those media which can be chased in dehydrated form. The use of dehydrated media saves time and gives ater uniformity between different batches.

For convenience in finding a particular formula, the media are listed habetically.

### tyl Methyl Carbinol Medium for Bacilli

| | |
|---|---|
| Proteose Peptone | 7 g. |
| Glucose | 5 g. |
| Sodium Chloride | 5 g. |
| Distilled Water | 1000 ml. |

Heat to dissolve. Adjustment of pH not necessary. Tube in 5 ml. amounts 18 mm. test tubes. Sterilize 15 min. at 121° C.

### onium Phosphate Arabinose Agar

| | |
|---|---|
| Monobasic Ammonium Phosphate ($NH_4H_2PO_4$) | 1 g. |
| Potassium Chloride | 0.2 g. |
| Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$) | 0.2 g. |
| Agar Agar | 13 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Add 12 ml. of 0.04% aque- Brom Cresol Purple. Tube in 5 ml. amounts. Autoclave at 121° C. for 15 . While still liquid add aseptically to the sterile medium, 0.25 ml. of ter-sterilized 10% aqueous arabinose solution. Mix. Slant tubes and let idify.

### onium Phosphate Glucose Agar

| | |
|---|---|
| Ammonium Phosphate ($NH_4H_2PO_4$) | 1 g. |
| Glucose | 10 g. |
| Potassium Chloride | 0.2 g. |
| Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$) | 0.2 g. |
| Agar Agar | 15 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Add 12 ml. of 0.04% aque- Brom Cresol Purple. Tube in 5 ml. amounts. Autoclave at 121° C. for 15 . Slant tubes and let solidify.

onium Phosphate Xylose Agar

See Ammonium Phosphate Arabinose Agar and substitute 0.25 ml. of filter-rilized 10% Xylose for the Arabinose. Otherwise the preparation is the same.

binose Broth Fermentation Medium

See B T B Fermentation Broth Base.

od Agar*

| | |
|---|---|
| Beef Heart, Infusion from | 500 g. |
| Bacto Tryptose | 10 g. |
| Sodium Chloride | 5 g. |
| Bacto Agar | 15 g. |
| Distilled Water, q.s. | 1000 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Sterilize in 100 ml. unts in 6-oz. prescription bottles for 15 min. at 121° C. (15 lbs/in$^2$). Coo 45° C. to 50° C. Add 5 ml. sterile defibrinated sheep, goat, or horse blood ptically. Mix by turning the bottle gently end over end. Distribute 8 to ml. per plate, or tube about 4 ml. amounts in sterile test tubes and slant Blood Agar slants.

B Fermentation Broth Base

| | |
|---|---|
| Beef Extract | 1 g. |
| Proteose Peptone No. 3 | 10 g. |
| Sodium Chloride | 5 g. |
| Brom Thymol Blue (1.6% alcoholic sol.) | 1 ml. |
| Distilled Water | 1000 ml. |

Heat to dissolve. Adjust to pH 7.0. For use, add 0.5% of desired ferment e substrate. Tube as fermentation tubes. Sterilize at 121° C. for 15 utes. Cool quickly at 45° C. in water. Remove, and when cooled to room perature, the medium is ready for use.

colate Agar (This base agar is called Bacto Proteose No. 3 Agar Dehydrated)

| | |
|---|---|
| Proteose Peptone No. 3 | 20 g. |
| Glucose | 0.5 g. |
| Sodium Chloride | 5 g. |
| Di Sodium Phosphate ($Na_2HPO_4$) | 5 g. |
| Agar Agar | 15 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve. Adjust to pH 7.4. Distribute in 100 ml. ounts in bottles. Sterilize at 121° C. for 15 minutes. Cool to 80° C. and d 5% by volume of fresh, sterile, defibrinated blood, and mix quickly but ntly. When the blood has turned chocolate brown in color, cool the medium 45° C. and pour into sterile Petri dishes or aseptically pipette into steri st tubes in 5 ml. amounts. Slant the tubes and let solidify.

rate Agar for Bacilli

| | |
|---|---|
| Potassium Acid Phosphate ($KH_2PO_4$) | 0.5 g. |
| Ammonium Nitrate | 2 g. |
| Sodium Citrate ($Na_3C_6H_5O_7 \cdot 5H_2O$) | 2 g. |
| Agar Agar | 13 g. |
| Tap Water | 1000 ml. |

Heat to boiling to dissolve. Adjust to pH 6.8. Add 10 ml. of 0.4% aque- Phenol Red solution. Tube in 5 ml. amounts. Sterilize 15 minutes at 121° C at tubes and let solidify.

rin Broth Fermentation Medium

See B T B Fermentation Broth Base.

itol Broth Fermentation Medium

See B T B Fermentation Broth Base.

lin Broth Fermentation Medium

See B T B Fermentation Broth Base.

ier Gelatin Agar (Modified)

| | |
|---|---|
| Nutrient Agar | 1000 ml. |
| Bacto Gelatin | 4 g. |

Heat to boiling to dissolve. Adjust to pH 7.0. Sterilize in 100 ml. unts in 6-oz. prescription bottles at 121° C. for 15 minutes. Cool to C. in water bath. Distribute each 100 ml. into 7 sterile Petri dishes.

cose Agar*

| | |
|---|---|
| Nutrient Agar | 1000 ml. |
| Glucose | 10 g. |

Heat to boiling to dissolve. Adjust to pH 7.0. Sterilize in flasks or es for 15 minutes at 121° C.

cose B C P Agar

| | |
|---|---|
| Glucose Agar | 1000 ml. |
| Brom Cresol Purple (4% alc. sol.) | 1 ml. |

Tube in 5 ml. amounts. Sterilize 15 minutes at 121° C. Solifify in an ight position.

cose Broth Fermentation Medium

## ucose Broth for Bacilli

| | |
|---|---|
| Glucose | 5 g. |
| Proteose Peptone | 10 g. |
| Distilled Water | 1000 ml. |

Heat to dissolve. Do not adjust reaction. Tube in 5 ml. amounts. rilize 15 minutes at 121° C.

## rcerol Agar for Pigment Production

| | |
|---|---|
| Potassium Citrate ($K_3C_6H_5O_7 \cdot H_2O$) | 1 g. |
| Di Ammonium Phosphate $(NH_4)_2HPO_4$ | 0.5 g. |
| Potassium Bicarbonate | 0.5 g. |
| Di Potassium Phosphate ($K_2HPO_4$) | 1.5 g. |
| Proteose Peptone | 20 g. |
| Agar Agar | 18 g. |
| Glucose | 0.5 g. |
| Glycerol | 50 ml. |
| Distilled Water | 950 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Tube in 5 ml. amounts. rilize at 121° C. for 15 minutes. Slant the tubes and let solidify.

## rcerol B C P Agar

| | |
|---|---|
| Nutrient Agar | 1000 ml. |
| Glycerol | 10 g. |
| Brom Cresol Purple (4% alcoholic sol.) | 1 ml. |

Tube in 5 ml. amounts. Sterilize 15 minutes at 121° C. Solidify in up- ght position.

## ycerol Broth Fermentation Medium

See B T B Fermentation Broth Base.

## ositol Broth Fermentation Medium

See B T B Fermentation Broth Base.

## ulin Broth Fermentation Medium

See B T B Fermentation Broth Base.

## ser's Citrate Medium*

| | |
|---|---|
| Sodium Ammonium Phosphate ($NaNH_4HPO_4 \cdot 4H_2O$) | 1.5 g. |
| Potassium Phosphate, Monobasic ($KH_2PO_4$) | 1 g. |
| Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$) | 0.2 g. |
| Sodium Citrate ($Na_3C_6H_5O_7 \cdot H_2O$) | 3 g. |

Dissolve all the ingredients. Adjust to pH 6.8. Tube in 5 ml. amounts.

ctose B C P Agar

| | |
|---|---|
| Nutrient Agar | 1000 ml. |
| Lactose | 5 g. |
| Brom Cresol Purple (4% alcoholic sol.) | 1 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Tube in 5 ml. amounts. erilize at 121° C. for 15 minutes. Solidify in an upright position.

ctose Broth Fermentation Medium

See B T B Fermentation Medium.

tmus Milk*

| | |
|---|---|
| Fresh Skim Milk | 1000 ml. |
| Litmus, powdered | 5 g. |

or

| | |
|---|---|
| Dry Skim Milk | 100 g. |
| Litmus, powdered | 5 g. |
| Distilled Water | 1000 ml. |

Dissolve all constituents. Dispense in 5 ml. amount in tubes. Sterilize minutes at 121° C.

effler's Blood Serum Slants*

| | |
|---|---|
| Beef Extract | 0.3 g. |
| Tryptose | 1 g. |
| Glucose | 0.5 g. |
| Sodium Chloride | 0.5 g. |
| Distilled Water | 100 ml. |

Dissolve all the ingredients. Cool to room temperature.

d:

| | |
|---|---|
| Fresh Beef Serum | 300 ml. |

Tube in 5 ml. amounts in screw-capped tubes. Close caps tightly. Lay a slanting position in the autoclave. Close the autoclave and exhaust lves tightly. Bring the steam pressure quickly to 15 lbs. per sq. inch. ld for 10 minutes. Then gradually let the entrapped air from the autoclave thout letting the pressure fall until all the air is exhausted. Then close e exhaust valve and heat for 15 more minutes at 15 lbs. pressure. Shut off e autoclave, and let the pressure fall very gradually.

ltose Broth Fermentation Medium

See B T B Fermentation Broth Base.

## nitol B C P Agar

| | |
|---|---|
| Nutrient Agar | 1000 ml. |
| Mannitol | 5 g. |
| Brom Cresol Purple (4% alcoholic sol.) | 1 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Sterilize in 5 ml. unts at 121° C. for 15 minutes. Solidify in an upright position.

## nitol Broth Fermentation Medium

See B T B Fermentation Broth Base.

## % Methylene Blue Milk

| | |
|---|---|
| Fresh Skim Milk | 1000 ml. |
| Methylene Blue | 1 g. |

or

| | |
|---|---|
| Dry Skim Milk | 100 g. |
| Methylene Blue | 1 g. |
| Distilled Water | 1000 ml. |

Mix all ingredients in the cold until well suspended. Heat to boiling il methylene blue is dissolved. Tube in 5 ml. amounts. Sterile 15 minutes 121° C.

## k-Agar Medium

Sterilize fresh, skim milk in 50 ml. amounts in 6-oz. prescription bottles 121° C. for 15 minutes.

Make a 2% Agar Agar solution as follows:

| | |
|---|---|
| Agar Agar | 20 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve the agar agar. No adjustment of pH is neces- y. Sterilize in 50 ml. amounts in 6-oz. prescription bottles at 121° C. for minutes.

Cool the melted agar agar solution and sterile skim milk to 45° C. Combine equal amounts and pour into sterile Petri dishes. When solidified it is dy for use.

## ility Test Agar Medium*

| | |
|---|---|
| Tryptose | 10 g. |
| Sodium Chloride | 5 g. |
| Bacto Agar | 5 g. |
| Distilled Water | 1000 ml. |

Dissolve the ingredients by boiling. Adjust to pH 7.4. Tube in 5 ml. unts. Sterilize 15 minutes at 121° C. Let solidify in an upright position.

-V.P. Medium*

| | |
|---|---|
| Proteose Peptone | 7 g. |
| Glucose | 5 g. |
| Di-Potassium Phosphate ($K_2HPO_4$) | 5 g. |
| Distilled Water | 1000 ml. |

Dissolve. Adjust to pH 7.0. Tube in 10 ml. amounts. Sterilize 15 minutes 21° C.

ogen-Free Mannitol Agar

| | |
|---|---|
| Mannitol | 15 g. |
| Di-Potassium Phosphate ($K_2HPO_4$) | 0.2 g. |
| Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$) | 0.2 g. |
| Calcium Chloride ($CaCl_2$) | 0.02 g. |
| Ferric Chloride ($FeCl_3 \cdot 6H_2O$)(10% sol.) | 0.05 ml. |
| Molybdenum trioxide (0.1% aq. sol.) | 1 drop |
| Distilled Water | 1000 ml. |
| Agar Agar | 15 g. |

Heat to boiling to dissolve. Adjust to pH 7.2. Dispense in 5 ml. amounts ubes or in 100 ml. amounts in bottles. Sterilize at 121° C. for 15 minutes. it the tubes and let solidify. The bottles should be cooled to 45° C. then tributed among 7 sterile Petri dishes for use.

rient Agar*

| | |
|---|---|
| Beef Extract | 3 g. |
| Peptone | 5 g. |
| Agar Agar | 15 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Tube in 5 ml. amounts or pense in 100 ml. amounts in 6-oz. bottles. Sterilize 15 minutes at 121° C. nt the tubes and let solidify.

9.6 Nutrient Agar Slants

Make like Nutrient Agar except pH is adjusted to pH 9.6 before steriliza- n. It is dispensed in 5 ml. amounts in tubes. Sterilize at 121° C. for 15 utes. Slant tubes and let solidify.

rient Broth*

| | |
|---|---|
| Peptone | 5 g. |
| Beef Extract | 3 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve. Adjust to pH 7.0. Reheat to boiling. Filter rough filter paper. Dispense in tubes in 5 ml. amounts. Sterilize at 121° C. r 15 minutes.

ient Gelatin*

| | |
|---|---|
| Nutrient Broth | 1000 ml. |
| Bacto Gelatin | 120 g. |

Heat to boiling to dissolve. Adjust to pH 7.0. Dispense in tubes in 5 ml. nts. Sterilize at 121° C. for 15 minutes. Solidify tubes in an upright tion.

one Iron Agar*

| | |
|---|---|
| Bacto Peptone | 15 g. |
| Proteose Peptone | 5 g. |
| Ferric Ammonium Citrate | 0.5 g. |
| Di-Potassium Phosphate ($K_2HPO_4$) | 1 g. |
| Sodium Thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$) | 0.08 g. |
| Agar Agar | 15 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve, adjust to pH 7.0. Tube in 5 ml. amounts, and ilize 15 minutes at 121° C. Solidify the tubes in an upright position.

one Water Medium with Fermentation Vials

| | |
|---|---|
| Bacto Peptone | 5 g. |
| Distilled Water | 1000 ml. |

Heat to dissolve. Adjust to pH 7.0. Tube in 5 ml. amounts. Add an in- ed fermentation vial to each tube, plug, and sterilize at 121° C. for 15 tes.

one Yeast Extract Broth (Called Bacto Micro Inoculum Broth, Dehydrated)*

| | |
|---|---|
| Yeast Extract | 20 g. |
| Proteose Peptone #3 | 5 g. |
| Glucose | 10 g. |
| Mono Potassium Phosphate ($KH_2PO_4$) | 2 g. |
| Distilled Water | 1000 ml. |

Dissolve all ingredients in the water. Adjust to pH 6.8. Dispense in ml. amounts in 16 to 20 mm. tubes. Sterilize for 15 minutes at 121° C.

nol Red Tartrate Agar*

| | |
|---|---|
| Peptone | 10 g. |
| Potassium Sodium Tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) | 10 g. |
| Sodium Chloride | 5 g. |
| Agar Agar | 15 g. |
| Phenol Red (0.04% aq. sol.) | 6 ml. |
| Distilled Water | 1000 ml. |

Heat to boiling until dissolved. Adjust to pH 7.5. Dispense in 10 ml. unts in tubes. Sterilize at 121° C. for 15 minutes. Solidify medium in an

## tato Slants

From sound, fresh, raw potatoes cut plugs with a cork borer with a diameter ightly smaller than the test tube to hold them. Cut off the end of each plug remove peel. Cut diagonally across the plug to give 2 wedge-shaped pieces of tato. Soak the cut plugs in cold running water. Insert a small pledget of sorbant cotton into the test tube. Moisten the cotton with 1 to 2 ml. of stilled water. Insert potato plug large end down. Sterilize at 121° C. for minutes. The slanting surface is used for stroke cultures.

## tassium Nitrate Broth*

| | |
|---|---|
| Peptone | 5 g. |
| Beef Extract | 3 g. |
| Potassium Nitrate | 1 g. |
| Distilled Water | 1000 ml. |

Dissolve all ingredients. Adjust to pH 7.0. Tube in 5 ml. amounts. terilize at 121° C. for 15 minutes.

## otassium Nitrate Broth Fermentation Medium*

See Potassium Nitrate Broth. When tubing the medium insert an inverted ermentation vial before sterilizing. This medium is for detection of Nitrogen as production from nitrates.

## affinose Broth Fermentation Medium

See B T B Fermentation Broth Base.

## hamnose Broth Fermentation Medium

See B T B Fermentation Broth Base.

## alicin Broth Fermentation Medium

See B T B Fermentation Broth Base.

## 5% Sodium Chloride Agar

Make Nutrient Agar and add 50 grams Sodium Chloride per liter. Tube in 5 ml. amounts. Sterilize at 121° C. for 15 minutes. Slant tubes and let solidify.

## 6.5% Sodium Chloride Agar

Make Nutrient Agar and add 65 grams Sodium Chloride per liter. Tube in 5 ml. amounts. Sterilize at 121° C. for 15 minutes. Let solidify in an up-right position.

## ɔdium Hippurate Broth

To 1000 ml. of Nutrient Broth add 10 grams Sodium Hippurate. Dissolve. djust to pH 7.0. Filter through filter paper. Tube in 5 ml. amounts. terilize at 121° C. for 15 minutes.

## ɔrbitol B C P Agar

| | |
|---|---|
| Nutrient Agar | 1000 ml. |
| Sorbitol | 5 g. |
| Brom Cresol Purple (4% alcoholic sol.) | 1 ml. |

Tube in 5 ml. amount. Sterilize 15 minutes at 121° C. Solidify in an ɔright position.

## ɔrbitol Broth Fermentation Medium

See B T B Fermentation Broth Base.

## tarch Agar*

| | |
|---|---|
| Nutrient Agar | 1000 ml. |
| Soluble Starch | 2 g. |

Dissolve by boiling. Adjust to pH 7.0. Dispense in 100 ml. amounts in -oz. bottles. Sterilize 15 minutes at 121° C. Cool to 45° C. Distribute ach 100 ml. among 7 sterile Petri dishes.

## tarch Fermentation Broth Medium

See B T B Fermentation Broth Base. Use soluble starch as the fermentable ubstrate in 0.5% concentration.

## Sucrose Fermentation Broth Medium

See B T B Fermentation Broth Base.

## Trehalose Fermentation Broth Medium

See B T B Fermentation Broth Base.

## Tryptone Broth

| | |
|---|---|
| Bacto Tryptone | 10 g. |
| Beef Extract | 3 g. |
| Sodium Chloride | 5 g. |
| Distilled Water | 1000 ml. |

Dissolve all ingredients. Adjust to pH 7.0. Tube in 5 ml. amounts. Sterilize at 121° C. for 15 minutes.

## rea Broth Medium*

| | |
|---|---|
| Yeast Extract | 0.1 g. |
| Mono Potassium Phosphate ($KH_2PO_4$) | 9.1 g. |
| Disodium Phosphate ($Na_2HPO_4$) | 9.5 g. |
| Urea | 20 g. |
| Phenol Red (0.04% aq. sol.) | 25 ml. |
| Distilled Water, cold | 975 ml. |

Dissolve all the ingredients in the cold water. Adjust to pH 7.0. Filter-terilize, and distribute aseptically in 3 ml. amounts into sterile tubes. In-ubate overnight at 37° C. to check sterility!

## rea Glucose Agar

Make Ammonium Phosphate Glucose Agar but omit the Ammonium Phosphate. ube in 4.5 ml. amounts. Sterilize 15 minutes at 121° C. Prepare a 10% aque-us solution of urea and sterilize it by filtration through a bacteriological ilter. Cool the melted agar base to 45° C. Add 0.5 ml. of the sterile urea olution. Mix by rotating between the palms of the hands and slant. Solidify. ncubate overnight at 37° C. to check sterility!

## ylose Broth Fermentation Medium

Make B T B Fermentation Base. Tube in 5 ml. amounts, with inverted ermentation vials in the tubes. Sterilize at 121° C. for 15 minutes. septically add to each tube 0.5 ml. of 10% filter-sterilized Xylose solution. ix and incubate overnight at 37° C. to check sterility!

## east Extract Glucose Agar (Called Bacto Micro Assay Culture Agar, Dehydrated)*

| | |
|---|---|
| Yeast Extract | 20 g. |
| Proteose Peptone No. 3 | 5 g. |
| Glucose | 10 g. |
| Mono Potassium Phosphate ($KH_2PO_4$) | 2 g. |
| Agar Agar | 10 g. |
| Distilled Water | 1000 ml. |

Heat to boiling to dissolve. No adjustment of pH is necessary. Tube in 10 ml. amount. Sterilize at 121° C. for 15 minutes. Solidify in an upright position. This medium can be melted and poured into plates for isolation of Lactobacillus species when incubated in 10% $CO_2$ atmosphere.

## Appendix II

# A COMPILATION OF REAGENTS EMPLOYED IN THE MANUAL

### cid Alcohol

| | |
|---|---|
| 95% Ethyl Alcohol | 100 ml. |
| Conc. Hydrochloric Acid | 3 ml. |

### lbert's Diphtheria Stain

| | |
|---|---|
| Toluidine Blue | 0.15 g. |
| Methyl Green | 0.02 g. |
| Glacial Acetic Acid | 1 ml. |
| Ethyl Alcohol (95%) | 2 ml. |
| Distilled Water | 100 ml. |

### lbert's Iodine Reagent

| | |
|---|---|
| Iodine Crystals | 2 g. |
| Potassium Iodide | 3 g. |
| Distilled Water | 100 ml. |

Dissolve the Iodine and KI in about 10 ml. of the water. When dissolved, lute with the remainder of the water.

### % Alcoholic Alpha Naphthol

| | |
|---|---|
| Alpha Naphthol | 5 g. |
| 95% Ethyl Alcohol | 100 ml. |

### nthony's Stain

| | |
|---|---|
| Crystal Violet | 1 g. |
| Distilled Water | 100 ml. |

### queous Nigrosin

| | |
|---|---|
| Water-soluble Nigrosin | 10 g. |
| Distilled Water | 100 ml. |

Immerse in boiling water bath for 30 minutes, then add

| | |
|---|---|
| Formalin (Full strength) | 0.5 ml. |

Filter through filter paper and store in small test tubes in 5 ml. amounts.

## are-Gil's Flagella Stain Mordant

| | |
|---|---|
| Tannic Acid | 10 g. |
| Aluminum Chloride ($AlCl_3 \cdot 6H_2O$) | 18 g. |
| Zinc Chloride | 10 g. |
| Basic Fuchsin | 1.5 g. |
| 60% Ethyl Alcohol | 40 ml. |

Dissolve the solids by grinding in a mortar by adding the alcohol small ounts at a time.

## methyl Alpha Naphthylamine Reagent

| | |
|---|---|
| Distilled Water | 714 ml. |
| Glacial Acetic Acid | 286 ml. |
| Dimethyl Alpha Naphthylamine | 6 g. |

Mix in the order listed.

## rlich's Reagent #I

| | |
|---|---|
| 95% Ethyl Alcohol | 95 ml. |
| Para Dimethyl Amino Benzaldehyde | 1 g. |
| Conc. Hydrochloric Acid | 20 ml. |

## rlich's Reagent #II

| | |
|---|---|
| Potassium Persulphate ($K_2S_2O_8$) | 5 g. |
| Distilled Water | 100 ml. |

This makes a saturated solution. Decant the clear solution.

## rric Chloride Reagent

| | |
|---|---|
| Distilled Water | 100 ml. |
| Conc. Hydrochloric Acid | 2 ml. |
| Ferric Chloride ($FeCl_3 \cdot 6H_2O$) | 12 g. |

Mix in the order listed.

## razier Gelatine Developer

| | |
|---|---|
| Distilled Water | 100 ml. |
| Conc. Hydrochloric Acid | 20 ml. |
| Mercuric Chloride | 15 g. |

Add the hydrochloric acid to about half of the water. Add the Mercuric hloride. Dissolve completely. Dilute with the remainder of the water.

## ...m's Iodine Solution

| | |
|---|---|
| Iodine Crystals | 1 g. |
| Potassium Iodide | 2 g. |
| Distilled Water | 300 ml. |

Dissolve the solids in about 20 ml. of the water. Dilute with the remainder the water.

## ...cker's Ammonium Oxalate-Crystal Violet Stain

### Solution A

| | |
|---|---|
| Crystal Violet | 2 g. |
| 95% Ethyl Alcohol | 20 ml. |

### Solution B

| | |
|---|---|
| Ammonium Oxalate | 0.8 g. |
| Distilled Water | 80 ml. |

After solids in each solution are dissolved, mix by adding Solution A to ...lution B. Filter through filter paper into smaller bottles before use.

## ...effler's Alkaline Methylene Blue Stain

### Solution A

| | |
|---|---|
| Methylene Blue | 0.3 g. |
| 95% Ethyl Alcohol | 30 ml. |

Dissolve completely.

### Solution B

| | |
|---|---|
| Distilled Water | 100 ml. |
| Potassium Hydroxide | 0.01 g. |

Dissolve the KOH then pour Solution A into Solution B with constant stir-...ng. Filter through filter paper into small bottles before use.

## ...ethyl Red Indicator

| | |
|---|---|
| Methyl Red | 0.1 g. |
| 95% Ethyl Alcohol | 300 ml. |
| Distilled Water | 200 ml. |

Dissolve methyl red in the alcohol. When completely in solution, dilute ...ith the distilled water.

## ...essler's Reagent

### Solution A

| | |
|---|---|
| Potassium Iodide | 50 g. |
| Distilled Water ($NH_3$-free) | 35 ml. |

## ssler's Reagent (continued)

### Solution B

| | |
|---|---|
| Mercuric Chloride | 12 g. |
| Distilled Water ($NH_3$-free) | 200 ml. |

### Solution C

| | |
|---|---|
| Potassium Hydroxide | 202 g. |
| Distilled Water | 400 ml. |

Make each solution separately. Solution B will be a saturated solution at om temperature. Slowly add Solution B to Solution A until a slight precipite persists. Add Solution C. Mix. Dilute to 1 liter and allow to clarify settling of any precipitates. Decant the supernatant solution into clean rex bottles. Seal tightly against ammonia fumes.

## enol Red Indicator

| | |
|---|---|
| Phenol Red | 0.02 g. |
| 95% Ethyl Alcohol | 100 ml. |

## tato Starch Paste

| | |
|---|---|
| Potato Starch | 1 g. |
| Distilled Water | 100 ml. |

Add about 10 ml. of the water to the potato starch and stir into a thin, mooth paste. Heat the remainder of the water to boiling. Slowly add the tarch suspension to the boiling water with vigorous stirring. Boil until olution clears and becomes a thin gel.

## afranin Counterstain

| | |
|---|---|
| Safranin O | 0.25 g. |
| 95% Ethyl Alcohol | 10 ml. |
| Distilled Water | 100 ml. |

Dissolve the Safranin in the alcohol. When completely dissolved pour into he distilled water with vigorous stirring. Filter through filter paper into mall bottles before use.

## ulfanilic Acid Reagent

| | |
|---|---|
| Distilled Water | 714 ml. |
| Glacial Acetic Acid | 286 ml. |
| Sulfanilic Acid | 8 g. |

Mix in order listed.

## ...l's Carbol Fuchsin

### Solution A

| | |
|---|---|
| Basic Fuchsin (90% dye content) | 0.3 g. |
| 95% Ethyl Alcohol | 10 ml. |

### Solution B

| | |
|---|---|
| Phenol | 5 g. or 4.66 ml. melted Phenol |
| Distilled Water | 95 ml. |

Prepare Solutions A and B separately and pour Solution A into Solution B ...adually with vigorous stirring. Filter through filter paper into small bottles ...fore use.

ent's Name: _____

of Organism: _____ Culture No. _____

ce: _____

MORPHOLOGY on (Medium): _____ after ____ days at ____ °C.

Shape of Vegetative cells: _____

Arrangement of Cells _____

Irregular Forms _____

Size of Cells _____ Flagella _____

Motility in Broth _____ Motility in Semi-Solid Agar _____

Condition of Sporangia _____ after ____ days at ____ °C.

Endospores (Shape) _____ Size of Spores: _____

Position in Sporangium _____

Heat Test result _____

NING CHARACTERISTICS:

| | Age of Cells at time of staining |
|---|---|
| Gram Reaction | |
| Acid Fast stain | |
| Capsule stain | |
| Granule stain | |

COLONIES: Age _____ @ ____ °C.

Form _____

Elevation _____

Surface _____

Margin _____

Density _____

Internal Structure _____

Chromogenesis _____

AGAR STROKE: Age _____ @ ____ °C.

Amount of Growth _____

Form _____

Consistency _____

Density _____

Chromogenesis _____

Medium _____

tion to Free $O_2$:

erature Relations: Grows best at _____ °C.

lase Test results:

| rbohydrate | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 | 21 |
|---|---|---|---|---|---|---|---|
| inose | | | | | | | |
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| ose | | | | | | | |
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| erol | | | | | | | |
| itol | | | | | | | |
| itol | | | | | | | |
| itol | | | | | | | |
| itol | | | | | | | |
| cin | | | | | | | |
| lin | | | | | | | |

| Litmus Milk: Observation | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 |
|---|---|---|---|---|---|---|
| Reaction | | | | | | |
| Acid Curd | | | | | | |
| Rennet Curd | | | | | | |
| Peptonization | | | | | | |
| Reduction of Litmus | | | | | | |
| Gas Production | | | | | | |

Starch hydrolysis

Casein hydrolysis

Gelatin hydrolysis

$KNO_3$ reduced to $KNO_2$ Gas

Indole produced

$H_2S$ produced

Acetyl methyl carbinol produced

Methyl Red test

Urea used as N source

Urea hydrolyzed

$NH_3$ used as N source

Hemolyzes blood cells

...t's Name: _____

...f Organism: _____ Culture No. _____

...e: _____

MORPHOLOGY on (Medium): _____ after ____ days at ____ °C.

Shape of Vegetative cells: _____

Arrangement of Cells _____

Irregular Forms _____

Size of Cells _____ Flagella _____

Motility in Broth _____ Motility in Semi-Solid Agar _____

Condition of Sporangia _____ after ____ days at ____ °C.

Endospores (Shape) _____ Size of Spores: _____

Position in Sporangium _____

Heat Test result _____

NING CHARACTERISTICS:

| | Age of Cells at time of staining |
|---|---|
| Gram Reaction | |
| Acid Fast stain | |
| Capsule stain | |
| Granule stain | |

COLONIES: Age _____ @ ____ °C.

Form _____

Elevation _____

Surface _____

Margin _____

Density _____

Internal Structure _____

Chromogenesis _____

AGAR STROKE: Age _____ @ ____ °C.

Amount of Growth _____

Form _____

Consistency _____

Density _____

Chromogenesis _____

Medium _____

...tion to Free $O_2$:

...erature Relations: Grows best at _____ °C.

...lase Test results:

| ...rbohydrate | Temp: °C. Days | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 7 | 10 | 14 | 21 |
| ...binose | | | | | | | |
| ...nnose | | | | | | | |
| ...ose | | | | | | | |
| ...cose | | | | | | | |
| ...tose | | | | | | | |
| ...rose | | | | | | | |
| ...tose | | | | | | | |
| ...halose | | | | | | | |
| ...finose | | | | | | | |
| ...lin | | | | | | | |
| ...trin | | | | | | | |
| ...rch | | | | | | | |
| ...cerol | | | | | | | |
| ...nitol | | | | | | | |
| ...bitol | | | | | | | |
| ...citol | | | | | | | |
| ...citol | | | | | | | |
| ...icin | | | | | | | |
| ...alin | | | | | | | |

| Litmus Milk: Observation | Temp: °C. Days | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 7 | 10 | 14 |
| Reaction | | | | | | |
| Acid Curd | | | | | | |
| Rennet Curd | | | | | | |
| Peptonization | | | | | | |
| Reduction of Litmus | | | | | | |
| Gas Production | | | | | | |

Starch hydrolysis
Casein hydrolysis
Gelatin hydrolysis
$KNO_3$ reduced to $KNO_2$ Gas
Indole produced
$H_2S$ produced
Acetyl methyl carbinol produced
Methyl Red test
Urea used as N source
Urea hydrolyzed
$NH_3$ used as N source
Hemolyzes blood cells

's Name: _____

f Organism: _____ Culture No. _____

: _____

MORPHOLOGY on (Medium): _____ after ____ days at ____ °C.

hape of Vegetative cells: _____

rrangement of Cells _____

rregular Forms _____

ize of Cells _____ Flagella _____

otility in Broth _____ Motility in Semi-Solid Agar _____

ondition of Sporangia _____ after ____ days at ____ °C.

ndospores (Shape) _____ Size of Spores: _____

Position in Sporangium _____

Heat Test result _____

ING CHARACTERISTICS:

| | Age of Cells at time of staining |
|---|---|
| ram Reaction _____ | _____ |
| cid Fast stain _____ | _____ |
| apsule stain _____ | _____ |
| ranule stain _____ | _____ |

COLONIES: Age _____ @ ____ °C.

orm _____

levation _____

urface _____

argin _____

ensity _____

nternal Structure _____

hromogenesis _____

AGAR STROKE: Age _____ @ ____ °C.

Amount of Growth _____

Form _____

Consistency _____

Density _____

Chromogenesis _____

Medium _____

ion to Free $O_2$:

rature Relations: Grows best at _____ °C.

ase Test results:

| bohydrate | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 | 21 |
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| Litmus Milk: Observation | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 |
|---|---|---|---|---|---|---|
| Reaction | | | | | | |
| Acid Curd | | | | | | |
| Rennet Curd | | | | | | |
| Peptonization | | | | | | |
| Reduction of Litmus | | | | | | |
| Gas Production | | | | | | |

Starch hydrolysis

Casein hydrolysis

Gelatin hydrolysis

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Indole produced

$H_2S$ produced

Acetyl methyl carbinol produced

Methyl Red test

Urea used as N source

Urea hydrolyzed

$NH_3$ used as N source

Hemolyzes blood cells

t's Name: _____

f Organism: _____ Culture No. _____

: _____

IORPHOLOGY on (Medium): _____ after ____ days at ____ °C.

hape of Vegetative cells: _____

rrangement of Cells _____

rregular Forms _____

ize of Cells _____ Flagella _____

otility in Broth _____ Motility in Semi-Solid Agar _____

ondition of Sporangia _____ after ____ days at ____ °C.

ndospores (Shape) _____ Size of Spores: _____

Position in Sporangium _____

Heat Test result _____

ING CHARACTERISTICS:

| | Age of Cells at time of staining |
|---|---|
| ram Reaction | |
| cid Fast stain | |
| apsule stain | |
| ranule stain | |

COLONIES: Age _____ @ ____ °C.

orm _____

levation _____

urface _____

argin _____

ensity _____

nternal Structure _____

hromogenesis _____

AGAR STROKE: Age _____ @ ____ °C.

Amount of Growth _____

Form _____

Consistency _____

Density _____

Chromogenesis _____

Medium _____

ion to Free $O_2$:

rature Relations: Grows best at _____ °C.

ase Test results:

| bohydrate | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 | 21 |
|---|---|---|---|---|---|---|---|
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| Litmus Milk: Observation | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 |
|---|---|---|---|---|---|---|
| Reaction | | | | | | |
| Acid Curd | | | | | | |
| Rennet Curd | | | | | | |
| Peptonization | | | | | | |
| Reduction of Litmus | | | | | | |
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Starch hydrolysis

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$NH_3$ used as N source

Hemolyzes blood cells

t's Name: _____

f Organism: _____ Culture No. _____

: _____

MORPHOLOGY on (Medium): _____ after ____ days at ____ °C.

hape of Vegetative cells: _____

rrangement of Cells _____

rregular Forms _____

ize of Cells _____ Flagella _____

otility in Broth _____ Motility in Semi-Solid Agar _____

ondition of Sporangia _____ after ____ days at ____ °C.

ndospores (Shape) _____ Size of Spores: _____

Position in Sporangium _____

Heat Test result _____

ING CHARACTERISTICS:

| | Age of Cells at time of staining |
|---|---|
| ram Reaction _____ | _____ |
| cid Fast stain _____ | _____ |
| apsule stain _____ | _____ |
| ranule stain _____ | _____ |

COLONIES: Age _____ @ ____ °C.

orm _____

levation _____

urface _____

argin _____

ensity _____

nternal Structure _____

hromogenesis _____

AGAR STROKE: Age _____ @ ____ °C.

Amount of Growth _____

Form _____

Consistency _____

Density _____

Chromogenesis _____

Medium _____

ion to Free $O_2$:

rature Relations: Grows best at _____ °C.

ase Test results:

| bohydrate | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 | 21 |
|---|---|---|---|---|---|---|---|
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| lin | | | | | | | |

| Litmus Milk: Observation | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 |
|---|---|---|---|---|---|---|
| Reaction | | | | | | |
| Acid Curd | | | | | | |
| Rennet Curd | | | | | | |
| Peptonization | | | | | | |
| Reduction of Litmus | | | | | | |
| Gas Production | | | | | | |

Starch hydrolysis

Casein hydrolysis

Gelatin hydrolysis

$KNO_3$ reduced to $KNO_2$ Gas

Indole produced

$H_2S$ produced

Acetyl methyl carbinol produced

Methyl Red test

Urea used as N source

Urea hydrolyzed

$NH_3$ used as N source

Hemolyzes blood cells

's Name: _____

f Organism: _____ Culture No. _____

: _____

ORPHOLOGY on (Medium): _____ after ____ days at ____ °C.

ape of Vegetative cells: _____

rangement of Cells _____

regular Forms _____

ze of Cells _____ Flagella _____

tility in Broth _____ Motility in Semi-Solid Agar _____

ndition of Sporangia _____ after ____ days at ____ °C.

dospores (Shape) _____ Size of Spores: _____

Position in Sporangium _____

Heat Test result _____

NG CHARACTERISTICS:

| | Age of Cells at time of staining |
|---|---|
| ram Reaction | |
| id Fast stain | |
| apsule stain | |
| ranule stain | |

COLONIES: Age _____ @ ____ °C.

orm _____

levation _____

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ensity _____

nternal Structure _____

hromogenesis _____

AGAR STROKE: Age _____ @ ____ °C.

Amount of Growth _____

Form _____

Consistency _____

Density _____

Chromogenesis _____

Medium _____

ion to Free $O_2$:

rature Relations: Grows best at _____ °C.

ase Test results:

| bohydrate | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 | 21 |
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| Litmus Milk: Observation | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 |
|---|---|---|---|---|---|---|
| Reaction | | | | | | |
| Acid Curd | | | | | | |
| Rennet Curd | | | | | | |
| Peptonization | | | | | | |
| Reduction of Litmus | | | | | | |
| Gas Production | | | | | | |

Starch hydrolysis
Casein hydrolysis
Gelatin hydrolysis
$KNO_3$ reduced to $KNO_2$ Gas
Indole produced
$H_2S$ produced
Acetyl methyl carbinol produced
Methyl Red test
Urea used as N source
Urea hydrolyzed
$NH_3$ used as N source
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n's Name: _____

f Organism: _____ Culture No. _____

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MORPHOLOGY on (Medium): _____ after ____ days at ____ °C.

hape of Vegetative cells: _____

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| rbohydrate | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 | 21 |
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Arrangement of Cells _____

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| arbohydrate | Temp: °C. Days 1 | 2 | 4 | 7 | 10 | 14 | 21 |
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| ulin | | | | | | | |
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| ycerol | | | | | | | |
| nnitol | | | | | | | |
| rbitol | | | | | | | |
| citol | | | | | | | |
| citol | | | | | | | |
| lcin | | | | | | | |
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